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PRINCIPAL INVESTIGATOR: Linda Noble

CONTRACTING ORGANIZATION: The Regents of the University of California at San Francisco
San Francisco, CA 94118-6215

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14. ABSTRACT

Purpose: We are evaluating efficacy of GM6001, a matrix metalloproteinase (MMP) inhibitor in a murine model of spinal cord injury (UCSF) and in dogs (Texas A & M, TAMU) that sustain naturally occurring spinal cord injuries resulting from spontaneous intervertebral disk herniation (IVDH).

Scope: These studies focus on efficacy of GM6001 in the context of an optimal therapeutic window and dependency on injury severity, using clinically relevant neurologic and urologic outcome measures.

Major findings:

- Spinal cord injury (SCI) in mice resulted in marked injury severity-dependent changes in locomotor and bladder function.
- GM6001 has an extended therapeutic window. When given up to at least 8 hours post injury, GM6001 resulted in injury severity dependent efficacy in a murine model of SCI. GM6001 treatment resulted in both neurologic and urologic benefit after a moderate level of SCI. This recovery was associated with a decrease in spinal cord lesion volume, greater spared white matter volume, and decreased muscle hypertrophy as assessed by bladder wall thickness. We further show that gelatinase activity is increased in the bladder after SCI. Thus, improved bladder function may be due at least in part to a more direct effect of GM6001 on the bladder wall by attenuating aberrant remodeling remodeling. In contrast to these beneficial effects seen after a moderate level of SCI, GM6001 did not rescue locomotor or bladder function in mice with severe SCIs.
- Pharmacokinetic study of GM6001 in 10 dogs supports the short-term safety of the drug. Plasma drug levels following a single dose are sustained at a significant level likely to result in MMP inhibition for approximately 72 hours, which support a single dose strategy in the clinical trial (Levine et al PLOS ONE, 2014, Appendix).
- Completed enrollment of 20 moderately injured dogs to serially characterize cystometry post-SCI. Compliance, capacity, residual volume, maximal voiding pressure, and post-voiding pressure was significantly different between healthy and injured dogs.
- Completed enrollment (90 of 90 dogs) for preclinical study comparing primary motor and cystometric endpoints in dogs treated with DMSO (vehicle) versus GM6001+DMSO. There were no significant differences in motor outcomes between treatment group 1 and group 0 (data still blinded). Group 1 dogs had significantly more adverse events and increased bladder compliance, a primary outcome measure of bladder function, compared to group 0 at day 42 post-SCI.

Significance: GM6001 is efficacious when the therapeutic window is extended up to at least 8 hours after murine SCI of moderate severity. The extended therapeutic window offers greater opportunity for translation to the theater for those soldiers who have sustained moderate SCIs. In dogs, one treatment arm had significantly greater number of adverse events and increased bladder compliance at day 42. Canine data will remain blinded until longitudinal analysis is complete.

15. SUBJECT TERMS

spinal cord injury, matrix metalloproteinase inhibitor, intervertebral disk herniation, mouse, dogs, urologic function, neurologic function

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INTRODUCTION

The primary objectives of this research are to evaluate the efficacy of a general inhibitor of matrix metalloproteinases, GM6001, in both a murine model of spinal cord injury (SCI) and in dogs who have sustained a naturally occurring SCI resulting from the sudden rupture of an intervertebral disk. The study builds upon our earlier work in a murine model of SCI, which showed that GM6001 significantly improved neurologic outcome when given 3 hours post injury after a moderate SCI (1). Thus, the goal here was to determine if GM6001 is likewise efficacious if the window of therapeutic intervention is extended and if the injury is more severe. An additional objective was to determine if GM6001 improves bladder function. We tested the efficacy of GM6001 dissolved in DMSO when administered subcutaneously at 8 hours after either a moderate or severe injury in mice. GM6001 improved both neurologic and urologic outcomes in the moderately injured group but not in the more severely injured group. Findings from the mouse studies have served to inform the dog preclinical trial, where the focus has been on the initial categorization of dogs according to severity of injury and assessment of GM6001 efficacy as determined by both neurologic and urologic assessments.

Please note that each task, described below, is indicated in bold. The requested and approved changes are indicated in bold italics. **Our most current findings in the dog studies are highlighted in yellow. Please note that we are still analyzing these data and so remain blinded to the groups- and as such the groups are designated as group 0 and group 1.**

BODY

UCSF Site:

Specific Aim 1

Task 1. Refine the therapeutic window for GM6001 in mice

1a. Obtain animal use protocol approval to study 165 mice (months 1-4)

We received approval from the UCSF IACUC and ACURO to conduct these studies.

1b. Compare neurologic recovery in 30 mice when GM6001 is initiated at 8 hours post injury. (months 5-6)

For reasons described below (Texas A & M, Specific Aim 2, Task 1b) GM6001 was not available until month 8th of this project. In the interim, we refined our murine SCI model so that we could reproducibly generate both moderate and severe SCIs (as required for Specific Aim 1, Task 2a) and defined a series of abnormal urologic parameters that are present after SCI including uninhibited bladder contractions and changes in peak bladder pressure, bladder volume, and bladder weight. These experiments have provided a foundation for Specific Aim 1, Task 3A. Finally, beginning in month 10, we began Task 1b. Below summarizes our findings.

To confirm a reproducible, graded model of SCI, male, C57Bl/6 mice were subjected to a 2 gm weight dropped 5 cm (mild injury), a 2 gm weight dropped 7.5 cm (moderate injury), or a 3 gm weight dropped 5 cm (severe injury) onto the cord exposed at the T 9 vertebral level (**Figure 1**). Severity was defined based upon the Basso Mouse Scale (BMS) where a score of 0 indicates hindlimb paralysis and a score of 9 reflects normal hindlimb locomotor function. The more mildly injured animals showed scores of about 7.5 (frequent to consistent stepping and mostly coordinated in their locomotion). The moderately injured group scored about 3.5 (occasional plantar stepping). The severely injured animals scored about a 2 (hindlimb movement limited to extensive ankle movement). Representative urodynamic tracings (**Figure 2**), resulting from awake cystometry in mice subjected to mild, moderate, or severe SCIs, revealed distinct differences between injury severities with mild injuries showing qualitatively the most prominent bladder contractions relative to the moderately and severely injured group.

We initially addressed long-term urologic status after mild and moderate SCIs, focusing on 4 measures- namely uninhibited bladder contractions (UICs), residual urine, bladder weight, and bladder volume. As might be expected mild and moderate injuries resulted in more prominent uninhibited bladder

contractions than sham controls (Kruskal–Wallis test followed by Dunn’s Multiple Comparison Test). **(Figure 3, Upper Panel)**. Residual urine **(Figure 3, Bottom Panel)** was similar between mild SCIs relative to shams but was elevated in the moderately injured group relative to shams (Kruskal-Wallis followed by Dunn’s Multiple Comparison Test). In contrast, peak voiding pressure showed no differences between mild and moderate SCIs and sham groups **(Figure 4)**. Finally, we analyzed bladder volumes and bladder weights using the same statistical approaches. Bladder volume remained unchanged, relative to shams, after a mild injury, whereas significantly increased after a moderate injury **(Figure 5)**. Bladder weight showed incremental increase in response to injury **(Figure 6)**.

In summary, we have successfully generated reproducible graded levels of injury severity based upon the BMS. Urologic status shows injury severity-dependent changes with UICs being most pronounced after a more mild injury than a moderate injury. We believe that reduced uninhibited bladder contractions with greater severity of SCI may reflect prolonged over distension of the bladder wall, which may damage the muscle layer. We further found greater residual urine, bladder volume and bladder weight in the moderate injured group relative to the sham controls. These findings suggest aberrant remodeling of the bladder wall, which could contribute to increased weight of this structure and reduced voiding.

We next evaluated the efficacy of GM6001 when give 8 hours after a moderate SCI, using a blinded, randomized experimental design with a priori exclusion criteria. A total of 25 C57Bl/6 adult male mice were subjected to a moderate SCI at T9. Two groups were studied: drug-treated (N= 12) or vehicle (carboxymethylcellulose)-treated (n= 13). At 8 hours post injury, all mice were evaluated using the BMS. Exclusion criteria were as follows: any animal showing an average score of > 0.5 at 8 hours post-injury or morbidity as defined in UCSF IACUC and ACURO approved protocols. GM6001, (100mg/kg, i.p.) was given at 8 hours post-injury followed by repeated administration every 12 hours for 3 days. The vehicle control group received the same timing/route of administration. Of the 12 GM6001 treated mice, a total of 5 were excluded from the study due to early death or early morbidity. Of 13 mice that were treated with vehicle, 4 were removed from study due to morbidity, 2 met exclusion criteria at 8 hours post injury, and 2 others had injury device malfunction. Thus, neurologic recovery was evaluated in N= 5 for the vehicle and N= 7 for drug. Two-way repeated measures ANOVA of the BMS score revealed the following: P= 0.58 for interaction, P<0.0001 for time, and P= 0.16 for treatment **(Figure 7)**.

We then evaluated improvement between the groups by comparing initial BMS scores at day 1 relative to final BMS scores at day 35 **(Figure 8)**. Based upon a Student T-test, the drug treated group shower greater improvement than the vehicle group (P= 0.025). Finally, since weight supported stepping is considered to be a very favorable outcome, we evaluated the percentage of mice that showed frequent stepping **(Figure 9)**. Statistical comparisons (2-way ANOVA) were done on percentages that were transformed into arcsin values. Approximately 60% of mice, treated with GM6001, showed frequent stepping whereas only 40% achieved that degree of recovery in the vehicle treated group. Based upon a 2-way ANOVA there was a significant effect of both treatment (P= 0.017) and time (P= 0.015). Taken together, despite the small group sizes, the behavioral data generally support improved recovery in mice treated with GM6001.

Finally, we have analyzed a cohort of bladders from these animals by awake cystometry. While we saw no differences in bladder volume, bladder weight or residual urine, the GM6001 treated group showed a significant reduction in uninhibited bladder contractions, one of the key features of dyssynergia **(Figure 10)**.

After approval from the UCSF IACUC and ACURO, we began a 2nd set of studies to assess efficacy of GM6001 using dimethyl sulfoxide (DMSO) as the vehicle and a subcutaneous (s.c) route of delivery. This change in experimental design was prompted by the design of the TAMU dog study, which required DMSO and s.c. drug delivery.

Task 1b. Compare neurologic recovery in 30 mice when GM6001 dissolved in DMSO and injected subcutaneously is initiated at 8 hours post injury. (Months 12-14)

We evaluated the efficacy of GM6001 dissolved in DMSO when given s.c at 8 hours after injury, using a randomized, blinded design with *a priori* exclusion criteria.

A total of 32 C57Bl/6 adult male mice were subjected to a moderate SCI at T9- and randomized to receive drug (n= 16) or vehicle (n= 16). At 8 hours post injury, all mice were evaluated using the BMS. Exclusion criteria were as follows: any animal showing an average score of > 0.5 at 8 hours post-injury (n=6) or morbidity as defined in UCSF IACUC and ACURO approved protocols (n=3) or an absence of locomotor recovery at 14 days post injury (n=1). GM6001 in DMSO was given s.c. at 100mg/kg at 8 hours post-injury followed by repeated administration every 12 hours for 3 days. The vehicle control group received the same timing/route of administration. Of the 16 GM6001 treated mice, 3 met exclusion criteria at 8 hours post-injury, 1 at 14 days post-injury and 1 had device malfunction. Of 16 mice that were treated with vehicle, 1 was removed from study due to morbidity, and 3 met exclusion criteria at 8 hours post injury. Thus, neurologic recovery was evaluated in N= 12 for vehicle and N=11 for drug group (**Figure 11**).

Neurologic recovery was measured using the BMS. Testing was performed at day 1, 3 and weekly thereafter for 5 weeks post-injury. Two-way repeated measures ANOVA of BMS scores revealed the following: $P = 0.2453$ for interaction, $P < 0.0001$ for time, and $P = 0.0397$ for treatment (**Figure 12A**). That is, all animals showed significant improvement in locomotor ability over time and there was a significant improvement in the drug-treated group on locomotor recovery. The average BMS score in the drug treated group at 35 days was 4.273 (indicating occasional plantar stepping), whereas the vehicle treated group had an average BMS score at 35 days of 3.292 (mice have the ability to plantar place, with or without support, but exhibit no plantar stepping). Based on these positive findings, this year, we further compared the percentage of mice between the two groups that had the ability to step at 35 days post-injury (**Figure 12B**). Seventy-three percent of the GM6001-treated mice had the ability to step as compared to only 33% in the DMSO-treated group (chi square analysis, $p = 0.029$). Our findings demonstrate that GM6001 improves locomotor function in a model of moderate contusion injury, even when initiation of treatment is delayed by 8 hours post-injury.

This year, we further evaluated weight changes across groups (expressed as a percentage weight change over time) in moderately injured mice treated with DMSO or GM6001 [Two-way repeated measures ANOVA of body weight revealed the following: $P = 0.0317$ for interaction]. Since we observed significant interaction, we conducted Sidak's multiple comparisons test to analyze within treatment group changes. In response to injury, while all mice in the vehicle treated group lost weight over the first week ($p < 0.05$ in vehicle treated group 3 day vs. pre-injury and $p < 0.001$ in vehicle treated group 7 day vs. pre-injury), similar weight loss was not evident in the GM6001 treated group ($p > 0.05$ in drug treated group at 3-28 days post-injury compared to pre-injury values). Furthermore, by 14 days all drug treated mice had gained weight and by 35 days they had gained significantly more weight as compared to pre-injury values ($p < 0.05$). In contrast, the vehicle treated group did not show weight gain until day 21 ($p < 0.05$) and at day 35, values were similar compared to pre-injury measures ($p > 0.05$). Between treatment group analysis (Sidak's multiple comparisons test) showed that weight change at 14 days post-injury was significantly different between drug and vehicle treated groups ($p < 0.05$) (**Figure 13**).

Taken together, the above findings serve as the 2nd independent study to validate GM6001 as a therapeutic for SCI. We have shown that when used in combination with the vehicle methylcellulose and given i.p. at a delayed time-point (8 hours post moderate injury), GM6001 improves long-term neurological recovery. In task 1b, we confirm neurological efficacy using a different route of administration (s.c.) and a different vehicle (DMSO). Using body weight as an index of overall health, we also found that mice treated with the drug were "healthier" post injury as compared to vehicle treated group.

Task 1c. Compare neurologic recovery in 30 mice when GM6001 is initiated at 6 or 12 hours depending on the results 1b. (Months 7-8)

We received permission from the Grants Office's Representative to eliminate task 1c, so that we could repeat task 1b, testing a subcutaneous route of administration. Please see above for the repeat dosing at 8 h with DMSO as vehicle and subcutaneous route of injection for moderate level injury severity.

Task 2. Determine if GM6001 will be efficacious after a more severe SCI in mice.

2a. Compare neurologic recovery in 30 mice after a severe SCI. (Months 16-18)

We evaluated the efficacy of GM6001, dissolved in DMSO, when given subcutaneously at 8 hours after severe SCI, using a blinded, randomized design with a priori exclusion criteria. A total of 31 C57Bl/6 adult male mice were subjected to a severe contusion injury at T9- Drug (N= 16) and vehicle (N= 15). At 8 hours post injury, all mice were evaluated using the BMS. Exclusion criteria were as follows: any animal showing an average score of > 0.5 at 8 hours post-injury or morbidity as defined in UCSF IACUC and ACURO approved protocols (n=2). GM6001 (100 mg/kg), dissolved in 99% DMSO, was given s.c. at 8 hours post-injury followed by repeated administration every 12 hours for 3 days. The vehicle control group received the same timing/route of administration. Of the 16 GM6001 treated mice, 1 had device malfunction and 3 met the morbidity criteria. Of 15 mice that were treated with vehicle, 2 were removed from study due to morbidity. Thus, neurologic recovery was evaluated in N= 12 for vehicle and N=13 for drug group.

Neurologic recovery was measured using the BMS. Testing was performed at day 1, 3 and weekly thereafter for 5 weeks post-injury. Two-way repeated measures ANOVA of the BMS score revealed the following: $P = 0.9468$ for interaction, $P < 0.0001$ for time, and $P = 0.7530$ for treatment (**Figure 14**). That is, all animals showed significant improvement in locomotor ability over time but there was no effect of drug on motor recovery. The average BMS score in both groups at 35 days was 2.6, which means extensive ankle movement.

Body weights were evaluated over time in severely injured mice treated with DMSO or GM6001. All animals gained weight with time, but there was no effect of drug treatment at this level of injury (Two-way repeated measures ANOVA: $P = 0.2662$ for interaction, $P < 0.0001$ for time, and $P = 0.5362$ for treatment) (**Figure 15**).

Task 3. Determine if GM6001, when optimally delivered, will improve bladder function in mice.

3a. Compare urologic function in spinal cord injured mice treated with either vehicle or GM6001 after moderate and severe SCIs. (Months 14-20)

At 5 weeks after a moderate or severe SCI in mice treated with GM6001 or DMSO, a PE10 catheter was implanted into the bladder dome and 2-3 days later awake cystometry was conducted. The following parameters were measured: time to first void, uninhibited bladder contractions/cycle, residual urine and voiding efficiency. Time to first void was defined as the period between when the saline infusion was initiated to the first release of fluid from the urethral meatus. To measure uninhibited bladder contractions (UIC), animals were first exposed to an equilibration period of 30 minutes during which the bladder was filled with saline. After this equilibration period, UICs were evaluated. UIC's were defined as rhythmic intravesical pressure rises (>5 cm H₂O from baseline pressure) without a release of fluid from the urethra using three representative voiding cycles. The numbers of non-voiding UICs per voiding cycle were determined. Residual urine was measured after the last void. The infusion was stopped and residual volume was determined by withdrawing the residual saline through the intravesical catheter. Voiding efficiency was calculated as $(\text{total infused volume} - \text{residual urine}) / \text{total volume} * 100$.

Cystometry results after moderate SCI. In the intact spinal cord, long descending fiber tracts from the midbrain coordinate the activity of the detrusor muscle and the urethral sphincter, i.e. the detrusor muscle is relaxed while the sphincter muscle is contracted to allow for filling of the bladder, while voiding is characterized by relaxation of the urethral sphincter and contraction of the detrusor muscle. After SCI, input from the brainstem is partially lost. As such, simultaneous contractions of detrusor and sphincter emerge. In cystometry, detrusor sphincter dyssynergia (DSD) is in part represented by detrusor contractions against a closed urethral sphincter without release of fluid (i.e. uninhibited bladder contractions). In addition to the parameters defined above, this year we also completed a detailed cystometry analysis that also included baseline pressure, threshold pressure, opening pressure, maximum pressure, intermicturition interval, duration of voiding, and amplitude of non-voiding contractions. We include here the data from uninjured mice that served as baseline controls and show the development of bladder dysfunction in injured mice. Furthermore using several parameters measured during awake cystometry, we show that GM6001 ameliorates the bladder dysfunction. Also included are cystometry tracings from representative mice from each of the groups. First, cystometry outcome values were established in a group of nine uninjured mice that served as baseline controls (**Table 1**). This

cohort demonstrated normal bladder function with short voiding duration and a small bladder capacity, as signified by the short time to first void. In addition, these animals showed a near perfect voiding efficiency with low intravesical post-void baseline values and minimal residual urine. An insignificant number of non-voiding contractions (NVCs) with small NVC amplitude were seen. All these parameters were altered in response to injury (compared to vehicle treated group, **Table 1**). GM6001 treatment ameliorates bladder dysfunction as compared to the DMSO-treated group by significantly increasing voiding efficiency (Mann-Whitney test, $p=0.010$) and thus decreasing post-void baseline pressure (Mann-Whitney test, $p=0.036$) and residual urine (unpaired t test, $t(20)=4.171$, $p=0.0005$) (**Figure 16, Table 1**). The time to first void/ leak significantly decreased (unpaired t test, $t(20)=2.836$, $p=0.010$), indicating that the bladder is less distended and hence the bladder capacity is lower. In addition, the number of NVCs per voiding cycle (Mann-Whitney test, $p=0.008$) and the amplitude (unpaired t test, $t(20)=2.307$, $p=0.032$) of NVCs was significantly decreased. **Figures 16E-G** show representative cystometry recordings for each of the study groups. NOTE: All the statistical analyses were run after checking data for normal distribution, data that were not normally distributed were compared by non-parametric test.

SCI-induced neurogenic bladder dysfunction leads to increased bladder weight and bladder wall thickness as a result of smooth muscle hypertrophy. For this study, bladder weight was normalized to body weight. The DMSO-treated group had greater bladder weight to body weight ratios than uninjured mice (unpaired t test, $t(19)=6.812$, $p<0.0001$). The bladder weight to body weight ratios were not significantly different between both treatment groups; however, there was a strong trend towards lower bladder to body weight ratios in the GM6001-treated group (Mann-Whitney test, $p=0.0509$) (**Figure 17A**). Since increased bladder weight may be a result of detrusor muscle hypertrophy, we next examined the bladder wall. The detrusor muscle thickness was assessed and compared separately in three regions: the bladder base, body, and dome. Comparing the DMSO-treated group to uninjured animals, there was a significant increase in detrusor muscle thickness at the base and body of the bladder (unpaired t tests; base: $t(9)=7.523$, $p<0.0001$; body: $t(9)=6.402$, $p=0.0001$; dome: $t(9)=1.969$, $p=0.0805$). Comparing the two treatment groups, the detrusor muscle diameter was less in each of the three regions in GM6001-treated mice as compared to DMSO-treated mice (unpaired t tests, base: $t(10)=3.191$, $p=0.010$; body: $t(10)=4.729$, $p=0.0008$; dome: $t(10)=3.041$, $p=0.013$) (**Figure 17B**). These findings suggest that GM6001 attenuates abnormal remodeling of the bladder wall after SCI, which mirrors the preservation of bladder function by the cystometry findings. **Figures 17C-H** show representative sections for each bladder region that is in each of the treatment groups.

Assessment of the ratio of collagen type III to collagen type I within the bladder detrusor muscle showed that there was a 40% loss in response to injury as compared to uninjured mice in the bladder base region (unpaired t-test, $t(9)=3.203$, $p=0.011$), a 38% loss in the body region (unpaired t-test, $t(9)=3.38$, $p=0.008$), and a 25% loss in the dome region (unpaired t-test, $t(9)=2.089$, $p=0.066$). This drop in collagen type III to collagen type I ratio was not alleviated by GM6001 treatment (base: $t(10)=0.183$, $p=0.859$; body: $t(10)=0.615$, $p=0.553$; dome: $t(10)=0.383$, $p=0.710$) (**Figure 18A**). Representative images from the bladder body region from uninjured, DMSO-treated, and GM6001-treated mice show the distribution of collagen type I and type III in the detrusor muscle (**Figures 18B-M**).

Given the favorable changes in bladder function, based upon awake cystometry in the GM6001-treated group, we considered the possibility that this may be attributed to a direct effect of the drug on the bladder wall. To address this hypothesis, we determined if gelatinase activity is expressed in the bladder wall. Using *in situ* zymography, we found that activity is apparent in the urothelium of the sham operated animals and that this activity appears to increase in a patchy distribution in the urothelium over time up to at least 7 days post injury (**Figure 19**). Thus, as GM6001, a nonspecific gelatinase inhibitor, is given over the first 3 days post injury there is opportunity to block this early rise in activity. Such blockade could alter the aberrant wound healing that contributes to bladder dysfunction after spinal cord injury.

Cystometry results after severe SCI. We have found that severe SCI results in a characteristic leakiness of the bladder, which likely results from a highly over distended bladder that with time disrupts the detrusor muscle.

Drug treatment did not affect urine retention (Unpaired two-tailed T- test, $p=0.6038$), time to first void (Unpaired two-tailed T- test, $p=0.9573$), or voiding efficacy (Mann Whitney test, $p=0.4401$) (**Figure 20**). However, it did result in a decreased number of UICs per cycle (Unpaired two-tailed T- test, $p=0.0210$). We believe that reduced UICs may reflect prolonged over distension of the bladder wall, which may

either damage the muscle layer or result in aberrant remodeling such that the muscle wall has reduced capability of contracting. Finally, we found that increased weight in the bladder was not attenuated by GM6001 (Unpaired two-tailed t-test, $p=0.1390$) (**Figure 21**).

Collectively, these findings support the position that GM6001 shows injury severity dependent efficacy whereby moderate levels of SCI show both neurological and urological recovery.

Task 4. Analysis of lesion epicenter and serotonergic fiber tracks caudal to a SCI in mice.

4a. Perfuse animals with fixative, remove the cords, and stain with Eriochrome cyanine or immunostain for serotonergic fiber tracks. (Months 5-24)

All animals thus far studied have been perfused with fixative, cryoprotected, frozen and sectioned. We have stained and analyzed spinal cord sections from all the mice from moderate and severe injured groups with eriochrome cyanine for measurement of residual white matter and lesion volume. After fixation and cryopreservation in sucrose, 1.5 cm of the cord, encompassing the epicenter was extracted. The cord was transected caudal and rostral with the epicenter in the middle, such that length of each segment is 5 mm. All three 5 mm long pieces were placed rostral to caudal in a square cryostat mold, flush against the right side of the mold and frozen in cryopreservation medium for sectioning. Serial 20 μm coronal sections were collected on 50 sequential Super-Frost slides, resulting in 15 sections per slide (5 per segment). An eriochrome cyanine (EC) staining protocol targeting myelin was developed, and every tenth slide was then stained. The section with the least amount of spared white matter was designated the lesion epicenter.

We have also stained half of the moderately injured mice to analyze serotonergic fiber tracks and developed stereological method of analysis for quantitation of fiber length.

4b. Quantify residual white matter and serotonergic fiber tracks caudal to injury. (Months 12-32)

The Eriochrome Cyanine stained spinal cord sections were analyzed in Stereo Investigator using the Cavalieri method. For analysis, a total of 15 sections per animal, at an interval of 200 μm apart were measured with epicenter section in the center, analysis was performed 1.4 mm in both rostral and caudal directions. The estimated total cord, spared white matter, and lesion volumes were determined. Volumetric analysis for the axial distribution of the lesion and spared white matter across the 3 mm segment was obtained and the percentage of spared white matter and lesion size relative to the total cord were calculated.

We analyzed the axial distribution of the lesion along a 3 mm segment with epicenter in the middle. To account for spinal cord size variability, lesion volume was normalized to cord volume and expressed as percentage lesion volume. For the moderately injured group, two-way repeated measures ANOVA of percentage lesion volume revealed the following: $P=0.0714$ for interaction, $P<0.0001$ for distance, and $P=0.0001$ for treatment (**Figure 22A**). That is, the size of the lesion decreased at distances further removed from the epicenter in both groups and there was an overall effect of treatment. Between groups comparisons (Sidak's multiple comparisons test) revealed significant effect of drug at 400 and 600 μm from epicenter. This shows that drug treated mice had overall smaller lesions as compared to the vehicle treated group.

We next analyzed the axial distribution of spared white matter in the moderately injured mice treated with either GM6001 or vehicle. Two-way repeated measures ANOVA of percentage spared white matter revealed the following: $P=0.1001$ for interaction, $P<0.0001$ for distance, and $P=0.0001$ for treatment (**Figure 22B**). That is, there was greater amount of spared white matter at distances further removed from the epicenter in both groups and there was an overall effect of treatment. Between groups comparisons (Sidak's multiple comparisons test) revealed significant effect of drug at 400 and 600 μm from epicenter. **Figures 22C and 22D** are reconstructions of the spinal cords of representative animal from the vehicle and drug treated group to demonstrate lesion (**Figures 22E and 22F**) and gray matter volumes (**Figures 22G and 22H**). This positive effect of drug on lesion volume and spared white matter was not apparent in the severely injured group (**Figures 23 and 24**).

We developed a method for unbiased stereological analysis to measure serotonergic fiber length, but due to limited number of sections that were available, we got a very high coefficient of error for our

analysis and will not be able to do this type of analysis for the moderately injured group that showed an effect of drug treatment.

4c. Statistically analyze data. (Months 30-36).

We have all completed statistical analyses of data that were collected at UCSF.

We are working on a manuscript on the murine spinal cord injury model to be submitted to Experimental Neurology for publication. In addition we are collaborating with group at Texas A & M to analyze the dog bladder cystometry data. Here we show the first analysis for residual urine comparisons in dogs enrolled in Phase 1 of the study (Figure 3, Supporting data from Texas A & M).

REPORT FROM Texas A & M

Specific Aims 2-3

Task 4. Measure MMPs in CSF in dogs

4a. Collect serum from dogs, conduct fluorogenic assays, and analyze data in approximately 125 dogs. (months 12-30)

Our group entered into a collaboration with Dr. Michael Heller at UC San Diego. Through that work, we have now been able to demonstrate that GM6001 has *in vitro* activity against MMP-2 and MMP-9 at concentrations that approximate those achieved in dog plasma (40-80 ng/mL) 72 hours following a single 100 mg/kg dose subcutaneously. These data together with the complete canine pharmacokinetics support the relevance of this strategy and also suggest that single dosing is likely adequate to achieve reasonably sustained MMP inhibition in dogs. We have already used these assays to analyze canine CSF and serum from dogs with SCI that were administered GM6001 in an NIH funded study. We have now been able to show serum elevation of MMP-2/MMP-9 following SCI and *in vitro* inhibition of serum MMP-2/MMP-9 3 days following delivery of a single 100 mg/kg dose of GM6001. Moreover, this novel assay will serve as a complementary approach to work at UCSF to address MMP activity in CSF using fluorogenic assays.

Additionally, we have begun a collaboration with Mayland Chang at University of Notre Dame to more critically examine metalloproteinase and ADAM activation following SCI. Using banked CSF from dogs with SCI not included in the DoD-funded trial, we have been able to show that the only active MMP detected is MMP-9 and the only active ADAM is ADAM-7 (see supporting data, Figure 1).

BODY

Texas A & M Site:

On 11/6/11 a sub-award agreement between UCSF and Texas A&M University (TAMU) was reached, permitting the ordering of materials to begin work at TAMU. Approvals for key purchases including urodynamics equipment (Laborie Goby), study drug (GM6001, SAI Advantium, India), and pharmacokinetic analysis (KCAS LLC, Kansas, USA) were obtained by mid-December 2011. Specific Aim 2, Task 1 was completed in July 2012, on schedule. Dog enrollment for Specific Aim 2, Task 2 and Specific Aim 3, Task 2 began in November 2012. Dog enrollment for specific Aim 3 task 1 began in July 2012. Enrollment of all dogs was completed July 2015 and data analysis was completed in May 2016.

Findings, partially supported by this funding, have been published in *PLOS ONE*. (Appendix)

Specific Aim 2

Task 1. Study of pharmacokinetics of GM6001 in 10 purpose bred dogs (months 1-12)

1a. Obtain animal use protocol approval at Texas A&M University (months 1-4)

Animal use protocols were approved at TAMU on 8/12/11

1b. Order GM6001 drug (months 1-4)

We were able to obtain permission through our Office of Sponsored Research (OSR) to order GM-6001 in mid-December 2011. A contract was executed with SAI Advantium and processing of the drug began in early January 2012. On March 22 2012, production of 110 g of GM-6001 at HPLC > 98% was completed. The drug was received at TAMU on 4/7/12. Unfortunately, delays associated with obtaining a sub-contract agreement, executing a contract with SAI, and actual drug production resulted in GM 6001 being available in month 8 of the study as opposed to the planned month 4.

1c. Order 10 purpose bred dogs (month 4)

Beagle-like dogs were obtained through the TAMU comparative medicine program in late April 2012, following the availability of GM6001. Dog purchase was delayed as a result of the delays in obtaining GM6001.

1d. Receive purpose bred dogs, allow for acclimatization (month 5)

Dogs were received and acclimatized by early May 2012.

1e. Perform physical examination and obtain complete blood count, chemistry, and urinalysis (month 5.5-6)

1f. Anesthetize dogs, place jugular catheters, and deliver GM6001 as a single 100 mg/kg subcutaneous dose (5 dogs) and two 100 mg/kg doses separated by 12 hours (5 dogs) (month 5.5-6)

(Figure 2, In Supporting Data)

1g. Serial serum acquisition (month 5.5-6)

Objectives 1e-1g were accomplished in mid-May 2012.

1h. Samples stored at -80C and shipped to PharmCats for gas chromatography (month 6)

KCAS was selected as an alternative vendor for pharmacologic studies as they had a lower bid than PharmCats and more rapid turn-around. Samples were shipped to KCAS in mid-May 2012.

1i. Samples processed by gas chromatography at PharmCats (months 6-10)

By mid-June 2012, KCAS generated pharmacokinetic data from dogs. These data were available within the anticipated time frame.

1j. Dr. Fajt to analyze pharmacokinetic data (months 10-12). Dr. Fajt will calculate drug elimination half life, peak drug concentration, time to peak concentration, area under the curve, and absorption half life. If serum levels remain elevated beyond the target duration of <5 in the single dose group, drug dose in the IVDH study population will be appropriately adjusted. If serum GM6001 levels are not present for at least 3 days with a single dose protocol, we will consider a 2 dose paradigm in the IVDH study population.

Dr. Fajt received pharmacokinetic data in mid-June 2012 and completed her analysis by July 1st 2012, 2 months ahead of the SOW schedule.

Summary Task 1: Delivery of GM6001 was accomplished in 10 purpose bred dogs. All dogs were clinically normal prior to drug administration based on physical examination, neurological examination, complete blood count, serum biochemistry, urinalysis, and CSF analysis. There were few adverse events associated with drug delivery: 10/10 dogs exhibited mild regional hyperesthesia at the delivery site which abated within 1-3 minutes and 10/10 dogs developed transient swelling at the delivery site. Swelling at the delivery site was 2-5 cm in diameter and at the time of the conclusion of the study had decreased in size to 1-3 cm. We have recognized similar swellings in a 4 dog safety study of GM6001

our group previously completed and in 35 dogs that have been administered the drug at 100 mg/kg S.C.

Analysis of the pharmacokinetics of GM6001 delivered S.C. in dogs suggests a rapid absorption and initial elimination followed by long-elimination half-life ("flip-flop phenomenon"). This pattern required a non-compartmental analysis. GM6001 was detected in plasma at the earliest time point following delivery (5 minutes) and had a mean time to maximal concentration (T_{max}) of 0.7 hours (S.D. +/- 1.3 hours). The mean maximal concentration (C_{max}) was 1370 ng/mL (S.D. +/- 361 ng/mL). The calculated elimination half-life for a single dose is 524 hours (S.D. +/- 428 hours). The mean concentration of GM6001 following single dose delivery was 80 ng/mL (S.D. +/- 20 ng/mL) at 96 hours. These data have been published in Levine et al PLoS ONE 2014.

Task 2. Compare motor recovery in dogs with IVDH (intervertebral disk herniation) associated SCI that receive saline placebo, DMSO vehicle, or GM 6001 (months 1-36)

2a. Obtain animal use protocol approval at Texas A&M University (months 1-4)

Animal use protocols were approved at TAMU on 8/12/11

2b. Obtain Clinical Research Review Committee approval (months 1-4)

Clinical Research Review Committee approval was granted at TAMU on 8/12/11

2c. Advertise clinical study via electronic brochures (months 6-18)

In February 2012 UCSF and TAMU began efforts to announce the study to media in order to develop interest in the general public. Stories were featured in the NY Times, ABC News, MS NBC, and on the Today Show website describing this unique collaboration. On June 1 2012 TAMU began efforts to advertise the study to our referring veterinarian population. These efforts included: 1) communications sent to a listserve ("Texasvets") comprised of veterinarians in July 2012, November 2012, February 2013, June 2013, August 2013, and February 2014 and 2) mailing electronic PDF brochures to referring veterinarians in November 2012, March 2013, March 2014, and February 2015.

2d. Advertise clinical study via referring veterinarian seminars (months 6-18)

We have held continuing education events that have featured this study at Veterinary Medical Associations in Montgomery County (September 2012 and February 2013), Washington County (January 2013), and Brazos County (December 2012 and October 2013).

2e. Advertise clinical study via print media (months 6-18)

The study was featured in our College's news magazine, "CVM Today" in October 2012 and again in summer 2014.

2f. Development of standardized databases (months 8-10)

Databases for the study were developed between January and February 2012. Data entry began at the start of enrollment.

2g. Enrollment of dogs with IVDH (months 13-30)

Enrollment was initiated in November 2012 (month 15). Enrollment was completed in July 2015, with 90/90 dogs included. A no-cost extension was required to complete this study. As of May 3 2016, data analysis is complete with the exception of a longitudinal analysis of motor scores and urodynamic variables.

Summary Task 2: Just prior to the beginning of enrollment, the study design was altered so that the saline control was eliminated; ACURO was contacted concerning this modification. This was done to enhance our power to detect differences between the DMSO group and GM6001 group. Enrollment has been completed. In May 2016, data analysis for this task was completed in 88 dogs (1 did not meet enrollment criteria and 1 had a subsequent injury during recovery – both excluded from analysis). While we have completed data analysis looking at our primary outcomes at the day 42 endpoint, we are still awaiting information from our statistician on a longitudinal analysis – therefore, we have remained blinded to group allocation. Data presented here reference the two treatment groups (0 and 1), although we do not know which one was treated with vehicle versus drug. The following were key findings:

- i. Baseline characteristics including age, breed, gender, injury severity at the time of enrollment as measured by a modified Frankel Score, lesion location, and T2 signal of the spinal cord on MRI did not differ significantly between treatment group 0 and treatment group 1. The absence of differences in baseline characteristics between groups indicated

that block randomization resulted in a population that was similar in each treatment arm. (Table 1)

- ii. Data were available at day 42 in 80/88 eligible dogs. The 8 dogs that did not have data available at day 42 were either lost to follow-up (n=4) or euthanized (n=4).
- iii. Dogs in treatment group 1 had significantly more adverse events (31/42; 74%) compared with dogs in group 0 (22/46; 48%) ($P = 0.0232$). The adverse events that were significantly more prevalent in group 1 compared to group 2 included injection site reactions (55% versus 15%; $P = 0.0005$) and musculoskeletal adverse events (14% versus 0%; $P = 0.0097$).
- iv. In the trial population of 80 dogs, initial injury severity as measured by a modified Frankel scale, age, Dachshund breed, and bladder capacity at admission were all significantly associated with 42 day TSCIS (our primary motor outcome) using a multivariate logistic regression model (See Table 2)
- v. Critically, treatment group was not significantly associated with day 42 TSCIS indicating that drug did not have an effect on motor performance.

Specific Aim 3

Task 1. Compare urodynamic measures in purpose bred dogs and dogs with IVDH (months 1-12)

1a. Obtain animal use protocol approval at Texas A&M University (months 1-12)

Obtained 8/12/2011

1b. Obtain Clinical Research Review Committee approval (months 1-4)

Completed 8/12/2011

1c. Order urodynamic equipment (month 1-4)

Urodynamic equipment was ordered in mid-December 2011 and arrived at TAMU in February 2011

1d. Order purpose bred dogs (month 4)

Purpose bred dogs were ordered in April 2012. As stated previously, this order was delayed due to delays in the production of GM6001.

1e. Receive purpose bred dogs, allow for acclimatization (month 5)

Purpose bred dogs were obtained and acclimatized. The acclimatization process was completed in early May 2012.

1f. Perform urodynamic studies in purpose bred dogs (month 6).

Ten purpose bred dogs will be utilized. Dogs will be sedated and will have a dual lumen urinary catheter, rectal catheter, and perineal volume following micturition will be recorded and voided volume and voiding efficiency will be calculated. Baseline pressure (vesical pressure after voiding), maximal voiding pressure (maximal vesical pressure during micturition) or leak point pressure (maximal vesical pressure in an animal without voluntary voiding, prior to urine overflow), voiding duration, and voiding interval (the frequency of voiding during filling) will be determined. The number of uninhibited bladder contractions will be recorded on each study. Finally, the timing of external anal sphincter EMG activity in relation to the voiding will be examined. Dogs with phasic contractions of the external anal sphincter during voiding that exhibit subsequent interrupted urine flow and elevated voiding pressure will be classified as having reflex dyssynergia. Voided volume and voiding efficiency will be calculated. Bladder ultrasound will be performed in all dogs immediately following voiding on the same days as urodynamic studies to determine residual urine volume. Animals will be placed in cages and provided water for 8 hours. Upon removal from the cage, dogs will be walked in a large outdoor area and allowed to voluntarily void without manual assistance. Immediately following voiding, an ultrasound machine will be used to measure transverse depth, transverse width, longitudinal length, longitudinal depth, and longitudinal width of the bladder. These measurements will be utilized to calculate residual bladder volume as has been previously described in dogs with IVDH.

Ten healthy beagle-like dogs were utilized to generate experimental data in early May 2012. At the outset of this study, it became clear that Ketamine sedation would be inadequate as it produces excessive spasticity in dogs, which may interfere with the assessment of urodynamic measures. We modified our AUP and received ACURO approval to utilize dexmedetomidine as an alternative sedative agent.

1g. Perform urodynamic studies in dogs with IVDH (months 6-12). A total of 25 dogs not enrolled in the GM6001 delivery trial will be utilized. Measurements will be performed at admission, and 3 days, 7 days, and 42 days following IVDH surgery. The same cystometric data as outlined in 1f will be recorded.

On June 1 2012 we opened enrollment to this clinical arm of the study. We have slightly modified inclusion criteria so that dogs lacking deep nociception are excluded due to the severity of the injury. Dogs lacking deep nociception have represented a small fraction (20%) of our IVDH associated SCI caseload and we did not believe that in a 25 dog population of dogs lacking deep nociception to make meaningful conclusions relative to typical urodynamic profile. The population of dogs included in this study was also changed to 20, to reduce animal use. Enrollment was completed in July 2015.

1h. Dr. Noah Cohen (VMD, PhD, MPH) has completed analysis for specific aim 3, task 1 and for the primary urodynamic outcomes at day 42 for task 2. We are in the process of performing a longitudinal analysis on our primary cystometric outcomes for task 2, therefore we have remained blinded to group identity as noted above.

Summary Specific Aim 3, Task 1: Cystometric measures were obtained in 10 purpose-bred Beagles and in 20 dogs with thoracolumbar IVDH that resulted in moderate SCI (non-ambulatory with intact nociception in the pelvic limbs). Examples of studies performed are provided in Figures 3 and 4 from TAMU site. Dogs tolerated sedated urodynamics well without reported adverse events. The following urodynamic variables were recorded: capacity, residual bladder volume, voiding efficiency, maximal bladder pressure (Pmax), maximal voiding pressure (Pvoid), and post-voiding pressure (Ppost). The primary urodynamic outcome was capacity. Because there were 3 to 4 runs per dog, and because the SCI dogs had repeated measures over time, it was necessary to account for the effects of clustering runs within dogs and dogs having repeated measures over time. This was accomplished using linear mixed-effects modeling, with a given urodynamic parameter (e.g., compliance) considered as the dependent (outcome) variable, individual dog modeled as a random effect (with or without run nested within dog), and with group-time variables modeled as fixed, categorical effects. The following was concluded:

Compliance was significantly higher among the SCI dogs relative to the control dogs at days 0, 3, and 7 ($P < 0.0001$ for each); however, the values were not significantly greater in the SCI dogs at 42 days than the controls ($P = 0.5268$). (Data presented in Figure 5)

Capacity was significantly higher among the SCI dogs relative to the control dogs at days 0 ($P < 0.0001$), 3 ($P < 0.0001$), and 7 ($P = 0.0062$ for each); however, the values were not significantly greater in the SCI dogs at 42 days than the controls ($P = 0.7094$).

Residual volume was significantly higher among the SCI dogs relative to the control dogs at days 0 ($P < 0.0001$) and 3 ($P = 0.0128$); however, the values were not significantly greater in the SCI dogs at 7 days or 42 days than the controls ($P = 0.0991$ and 0.8743 , respectively).

Efficiency did not differ significantly among controls and SCI dogs at any time, or among SCI dogs by time.

Voiding Pressure was significantly lower among the SCI dogs relative to the control dogs at days 0 ($P < 0.0001$), 3 ($P < 0.0001$), 7 ($P < 0.0001$), and 42 ($P = 0.0047$).

Maximal Pressure was significantly lower among the SCI dogs relative to the control dogs at days 0 ($P < 0.0001$), 3 ($P = 0.0001$), 7 ($P = 0.0063$), and 42 ($P = 0.0168$).

Post-voiding pressure did not differ significantly among controls and SCI dogs at any time, or among SCI dogs by time.

Task 2: Compare urodynamic measures in dogs with IVDH enrolled in the GM 6001 delivery trial (months 13-30).

Enrollment for this arm of the study started in month 15 and has been completed as of July 2015. Our primary urodynamic outcome at day 42 was determined a priori based on specific aim 3, task 1 results. The outcome selected was bladder compliance. This variable was selected principally due to limited variability within control and injured dogs in task 1 at test points and common use as a primary outcome in canine and human clinical trials. The following is a summary of urodynamic results from the 88 dog clinical trial comparing treatment group 1 and group 0. Note that urodynamic data were available in 80/88 dogs at day 42. Animals not evaluated at day 42 were either euthanized (n=4) or lost to follow up (n=4).

- i. A multivariable generalized linear regression model was used to assess factors associated with day 42 compliance. Weight > 7 kg, bladder capacity <75 mL at admission and treatment group 1 were associated with significantly increased bladder compliance. (Table 3)
- ii. Thus, there is a significant effect of treatment group on our primary urologic outcome.

KEY RESEARCH ACCOMPLISHMENTS

UCSF Site:

- Developed reproducible models of graded SCIs in the mouse.
- Defined key parameters to assess urologic status in mice after SCI
- Conducted the first study to assess efficacy of GM6001 when delivered 8 hours after a moderate SCI injury in mice. Though group sizes were small, these data showed promising results in terms of improving neurologic and urologic function.
- Completed all proposed studies in mice to assess efficacy of GM6001 when delivered 8 hours after two levels of injury severities. Here we mirrored the delivery route and vehicle to the dog clinical trial, so drug was dissolved in DMSO and injected subcutaneously.
- We show therapeutic efficacy of GM6001 in mice with a moderate level of spinal cord based upon both neurological and urological outcomes. However, similar efficacy as not seen after a severe spinal cord injury.
- Completed data analysis of other parameters of bladder function collected during the awake cystometry to assess bladder function in the moderately injured group treated with vehicle or GM6001 along with uninjured mice that served as baseline controls.
- Conducted 3D reconstruction of representative spinal cords from the moderately injured -vehicle and GM6001 treated mice.
- Completed histological analysis of the bladder wall thickness of the moderately injured -vehicle and GM6001 treated mice.
- Performed in situ gelatinase assay on fresh bladder sections from uninjured and 7 days post-injury.
- Developed unbiased stereology assay to assess serotonergic fiber length in the injured spinal cord.
- Stained bladders for collagen I and III and developed assay and analyzed bladders from moderately injured mice treated with vehicle or GM6001.

TAMU Site:

- Completed pharmacokinetic study of GM6001 in 10 dogs. The study supports the rapid development of maximal plasma concentration after S.Q. delivery, the presence of plasma drug levels capable of inhibiting MMPs in vitro, and the short term safety of the drug. Plasma drug levels following a single dose are sustained at a significant level likely to result in MMP inhibition for approximately 72 hours, which support a single dose strategy in the clinical trial. Data published in Levine et al PLoS ONE 2014.
- Completed normal dog cystometry in 10 dogs and injured dog cystometry in 20 dogs. Several cystometric variables were different between controls and affected dogs at multiple post-injury time points.
- Enrolled 90 dogs with acute IVDH-associated SCI into clinical trial (Specific Aims 2.2 and 3.2). Data analysis for primary outcomes at day 42 is complete. We are awaiting longitudinal analysis of primary outcomes.

REPORTABLE OUTCOMES

UCSF Site:

Poster presentation at the MHSRS/ATACC meeting, August 13-16, 2012 in Fort Lauderdale. Abstract was entitled "URINARY BLADDER DYSFUNCTION IN A MURINE MODEL OF SPINAL CORD INJURY: RELATIONSHIP BETWEEN INJURY SEVERITY AND MEASURES OF UROLOGIC STATUS". Abstract is provided in the Appendices.

Invited speaker, International Symposium on Neuroregeneration, December 7, 2011, Asilomar, CA
MATRIX METALLOPROTEINASES (MMPs) AND SPINAL CORD INJURY

Invited speaker, Ohio State University, January 14, 2013, Columbus, Ohio
MATRIX METALLOPROTEINASES AS A THERAPEUTIC TARGET FOR SPINAL CORD INJURY

Invited speaker, Rutgers University, W.M. Keck Center for Collaborative Neuroscience January 31, 2013, Piscataway, New Jersey
MATRIX METALLOPROTEINASES AS A THERAPEUTIC TARGET FOR SPINAL CORD INJURY

Invited speaker, American Veterinary Medical Association, July 19-23, 2013, Chicago, IL
TARGETING MATRIX METALLOPROTEINASES TO IMPROVE NEUROLOGICAL OUTCOME AFTER SPINAL CORD INJURY: A PATHWAY OF DISCOVERY FROM MICE TO DOGS
Abstract is provided in the Appendices.

Invited speaker, University of California, September 10, 2013, San Francisco, CA
TARGETING MATRIX METALLOPROTEINASES TO IMPROVE NEUROLOGICAL OUTCOME AFTER SPINAL CORD INJURY. A PATHWAY OF DISCOVERY FROM MICE TO DOGS

Presenter at nanosymposium, Society for Neuroscience annual meeting, November 9-13, 2013, San Diego, CA. Abstract was entitled, "ACUTE TREATMENT WITH THE MATRIX METALLOPROTEINASE INHIBITOR GM6001 IMPROVES LONG-TERM LOCOMOTOR AND BLADDER FUNCTION IN A MURINE MODEL OF SPINAL CORD INJURY". Abstract is provided in the Appendices.

Poster presentation at International Symposium on Neuroregeneration, December 11-15, 2013, Asilomar, CA. Abstract was entitled, "MATRIX METALLOPROTEINASES AS A THERAPEUTIC TARGET TO IMPROVE NEUROLOGICAL AND UROLOGICAL FUNCTION AFTER SPINAL CORD INJURY". Abstract is provided in the Appendices.

Poster presentation at the American Urology Association Meeting, May 16 - 21, 2014 in Orlando, Florida. Abstract was entitled "A MURINE MODEL OF SPINAL CORD INJURY: EFFECT OF THE GENERAL MATRIX METALLOPROTEINASE INHIBITOR GM6001 ON LONG-TERM LOCOMOTOR AND BLADDER FUNCTION". Abstract is provided in the Appendices.

Poster presentation at the National Neurotrauma Society Symposium, June 29-July 2, 2014 in San Francisco, CA. Abstract was entitled "MATRIX METALLOPROTEINASES AS A THERAPEUTIC TARGET FOR SUPPORTING UROLOGIC RECOVERY IN A MURINE MODEL OF SPINAL CORD INJURY". Abstract is provided in the Appendices.

TAMU Site:

Levine JM et al EFFICACY OF A MATRIX METALLOPROTEINASE INHIBITOR IN SPINAL CORD INJURED DOGS. PLOS ONE 2014; 9:e96408. Paper in pdf format is provided in the Appendices

Levine JM. Of Mice and Dogs. American College of Veterinary Internal Medicine Forum, June 2013.

Levine JM. Canine Intervertebral Disk Herniation: Translational Model. International Workshop of Veterinary Neuroscience, Hannover, Germany, February 2016.

CONCLUSIONS

- In a preliminary study, GM6001 (dissolved in 4% carboxy methyl cellulose and delivered via intraperitoneal route) when given 8 hours after a moderate SCI in the mouse, results in improvement in long-term neurologic recovery and a significant reduction in abnormal bladder contractility.
- Studied efficacy of GM6001 when delivered subcutaneously at 8 hours after moderate and severe SCI in the mouse. GM6001 treatment resulted in a long-term improvement in locomotor function and increased percentage of mice with the ability to step. Histological analysis of the injured spinal cord showed greater sparing of white matter with a corresponding reduction in lesion volume in the moderate injury group treated with GM6001. Awake cystometry revealed bladder dysfunction in the moderately injured mice treated with vehicle, which was ameliorated by drug treatment, as measured by a reduction in residual urine, uninhibited bladder contractions, reduction in time to first void and increased bladder efficiency. This was confirmed by histological analysis of the bladder, where injury led to increased bladder wall thickness and a significant reduction was observed in the GM6001 treated group relative to the vehicle control. In addition, to sparing white matter tracts, GM6001 may also act directly on the bladder urothelium, as seen by increased gelatinase activity at 7 days post-injury in untreated bladder.
- In contrast to these beneficial effects of GM6001 seen in the moderate injury group, there were no differences in neurological and urological function between GM6001 and vehicle-treated groups that sustained severe SCIs.
- GM6001 dosed subcutaneously at 100 mg/kg in dogs is safe and results in a pharmacokinetic profile that lends itself to the duration of MMP inhibition demonstrated to be effective in rodent neurotrauma work.
- In dogs with IVDH associated SCI, urinary voiding impairment can be assessed by cystometry and bears similarity to what is seen in humans with per-acute injury. Voiding recovery happens rapidly in dogs with mild or moderate SCI (non-ambulatory with or without limb movement but with intact deep nociception).
- In dogs included in the clinical trial, treatment group 1 was associated with significantly more adverse events compared to treatment group 0. Treatment group did not affect the primary motor outcome (day 42 TSCIS; a validated ordinal gait score). In contrast, dogs in treatment group 1 had significantly greater day 42 compliance (a primary urologic outcome) compared to group 0.

REFERENCES

1. Noble LJ et al *Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events. Journal of Neuroscience* 22, 7526 (2002)

APPENDICES:

Poster presentation at the MHSRS/ATACC meeting, August 13-16, 2012 in Fort Lauderdale.

TITLE: Urinary bladder dysfunction in a murine model of spinal cord injury: Relationship between injury severity and measures of urologic status

Thomas M. Fandel, Jonathan Levine, Kayleen Gimlin, Haoqian Zhang,
Alpa Mahuvakar, and Linda J. Noble-Haeusslein

Presenter's Name: Linda J. Noble-Haeusslein, Ph.D.

PURPOSE/AIMS: The purpose of this study was to determine the extent to which severity of an incomplete spinal cord injury (SCI) influences bladder function in a murine model of SCI.

DESIGN: Mice were randomized to sham, (n=8), mild (n=5) or moderate (n=7) SCI and treated with Enrofloxacin for 10 days subcutaneously followed by food supplemented with Enrofloxacin until euthanasia. Neurological status was evaluated at 1 and 3 days post injury and weekly thereafter for 3 weeks. At 4 weeks post-injury, awake cystometry was performed (n= 3-7/group). At the completion of cystometry and after residual urine was determined (n=3-6/group) the bladders were removed and weighed (n= 5-7/group). All observers were blinded to the experimental condition.

POPULATION/SAMPLE STUDIED: Adult, male, C57Bl6 mice subjected to laminectomy only or mild or moderate SCI.

METHODS: SCI was produced by dropping either a 2 g (mild injury) or 3 g (moderate injury) weight onto the exposed spinal cord at the T9 vertebral level. Neurological status was based upon the BMS scale. At 3 weeks post-injury, a PE50 polyethylene catheter was implanted into the bladder dome and tunneled subcutaneously to emerge in the interscapular area. One week later, cystometry was performed in the awake restrained animal using saline at an infusion speed of 16-20 ml/ minute (Catamount Research, St. Albans, VT). Residual urine was determined at the end of cystometry. The urinary bladders were removed, blotted dry, and weighed.

DATA ANALYSIS: Two-way repeated measures (RM) analysis of variance (ANOVA) was used to evaluate neurological recovery. Residual urine and bladder weight were analyzed using 1-way ANOVA followed by Bonferroni's Multiple Comparison Test. Unpaired Student's T-test was used when two groups were specified. Significance was defined at $P \leq 0.05$. All data are expressed as means \pm SEM.

FINDINGS: BMS scores revealed an effect of both time ($p=0.0001$) and injury severity ($p=0.0182$). While both injury groups showed improved performance over time, BMS scores were lower in the 3 g (1.786 ± 0.3595) relative to the 2 g (6.000 ± 1.508) group ($p=0.0097$) at 21 days post injury. Moreover, a 3 g injury led to qualitatively more uninhibited bladder contractions and greater residual urine (0.9293 ± 0.1346) and bladder weight (0.1475 ± 0.2238 g) relative to residual urine (0.3980 ± 0.0080) and bladder weight in the 2 g injury (0.07160 ± 0.0072 g) ($p < 0.01$).

CONCLUSIONS/RECOMMENDATIONS: There are injury severity dependent abnormal changes in both weight and function of the urinary bladder after SCI.

IMPLICATIONS: While bladder dysfunction is a common problem in human SCI, analyses of bladder function are typically neglected in murine models of SCI. Characterization of bladder function, relative to injury severity, provides a clinically relevant benchmark for establishing efficacy of candidate therapeutics.

FROM/TO TIME PERIOD OF STUDY: From September 30, 2011 to April 25, 2012

FUNDING: DOD Spinal Cord Injury Program SC100140

Invited speaker, American Veterinary Medical Association, July 19-23, 2013, Chicago, IL

TARGETING MATRIX METALLOPROTEINASES TO IMPROVE NEUROLOGICAL OUTCOME AFTER SPINAL CORD INJURY: A PATHWAY OF DISCOVERY FROM MICE TO DOGS

Presenter's Name: Linda J. Noble-Haeusslein, Ph.D.

Dogs sustain naturally occurring spinal cord injuries (SCIs) represent a clinically relevant population to confirm therapeutic targets that have been identified in rodent models of spinal cord injury (SCI). Spinal cord injured mice, genetically deficient in the gelatinase MMP-9 or treated with GM 6001, a broad-spectrum inhibitor of matrix metalloproteinases (MMPs), beginning 3 hours post injury, show improved long-term neurological outcomes that correspond to the early reduction of leukocytes in the injured cord and stabilization of the blood-spinal cord barrier. Here we find that serum levels of gelatinases are acutely elevated in spinal cord injured dogs, suggesting that these proteases may likewise be a determinant of recovery. After confirming safety and defining the pharmacokinetics of GM 6001 in normal dogs, a large scale, randomized, placebo controlled study was performed in dogs with acute SCIs. Duration of SCI was required to be ≤ 48 hours and dogs were stratified according to injury severity. Three groups were studied: GM 6001 + DMSO, DMSO, or saline. As GM 6001 is not soluble in an aqueous solution, DMSO was selected as the vehicle, recognizing that this agent has broad anti-inflammatory actions in models of CNS injury. Only the GM 6001-treated group, given shortly after admission to the clinic, resulted in a reduction in serum gelatinase activity. Utilizing post-hoc statistical techniques, there was a therapeutic benefit of GM 6001+DMSO over DMSO or saline in dogs with mild-moderate spinal cord injuries. These encouraging findings provide the first evidence that MMPs are a determinant of recovery after SCI in dogs. Such validation in a 2nd species reinforces the adverse interactions of these proteases in SCI, and suggests that GM 6001 may likely hold promise for human SCI.

Presenter at Nanosymposium, Society for Neuroscience annual meeting, November 9-13, 2013, San Diego, CA.

ACUTE TREATMENT WITH THE MATRIX METALLOPROTEINASE INHIBITOR GM6001 IMPROVES LONG-TERM LOCOMOTOR AND BLADDER FUNCTION IN A MURINE MODEL OF SPINAL CORD INJURY.

T. M. FANDEL, A. TRIVEDI-MAHUVAKAR, K. GIMLIN, H. ZHANG, J. M. LEVINE, A. F. MARTINEZ, L. J. NOBLE-HAEUSSLEIN

Presenter's name: Thomas M. Fandel

We have previously shown that the general matrix-metalloproteinase inhibitor GM6001, when given beginning 3 hours after moderate spinal cord injury (SCI), attenuates secondary injury to the murine spinal cord and improves locomotor recovery. Here we determine efficacy of GM6001 on locomotor and bladder function after more severe levels of SCI and when the initial treatment is delayed to 8 hours post injury. Adult, male, C57Bl/6 mice were subjected to moderate-severe (n=23) or severe (n=25) SCI, produced by dropping either a 2g or 3g weight respectively onto the exposed spinal cord at the T9 vertebral level. Injured mice were randomized to receive drug (GM6001 at 100mg/kg, n=11/12) or vehicle (99% DMSO, s.c. n=12/13) starting 8 hours after injury and then twice daily for 3 consecutive days. Neurological status was evaluated at 1 and 3 days post-injury and weekly thereafter for 5 weeks using the Basso Mouse Scale (BMS). At 5-6 weeks, a PE10 catheter was implanted into the bladder dome and conscious cystometry was performed 2-3 days later. Two-way repeated measures analysis of variance and unpaired T-Tests were used to evaluate locomotor recovery and bladder function, respectively. Significance was defined as $p < 0.05$. All observers were blinded to the experimental conditions. There was an effect of both time ($p < 0.0001$) and treatment ($p = 0.04$) on BMS score in the moderate-severe injury group. In contrast, in the severe injury group, there was an effect of time ($p < 0.0001$) but not of treatment on locomotor recovery ($p = 0.08$). Cystometry after moderate-severe injury revealed a therapeutic effect of GM6001 on post-void baseline pressure ($p = 0.01$), residual urine ($p = 0.0004$), the duration of the intermicturition interval ($p = 0.004$), the number of uninhibited bladder contractions (UBC)/voiding cycle ($p = 0.01$), and the pressure increase of UBC ($p = 0.01$). GM6001 treatment in the severe injury group had a positive effect on certain metrics; namely, the duration of the intermicturition interval ($p < 0.05$), the number of UBC/voiding cycle ($p = 0.004$), and the pressure increase of UBC ($p = 0.02$). Here we provide the first evidence that GM6001 improves both locomotor and bladder function when treatment is delayed to a more clinically relevant time point of 8 hours and after a moderate severe spinal cord injury. Such findings support the candidacy of this drug for clinical trials. While GM6001 did not improve locomotor function after a severe SCI, we are encouraged by the benefit seen in bladder function, which we speculate may result from the direct action of this drug on the bladder wall.

Poster presentation at International Symposium on Neuroregeneration, December 7, 2013, Asilomar, CA
CA

**MATRIX METALLOPROTEINASES AS A THERAPEUTIC TARGET TO IMPROVE NEUROLOGICAL
AND UROLOGICAL FUNCTION AFTER SPINAL CORD INJURY.**

A Trivedi, T. M. Fandel, A. F. Martinez, H. Zhang, J. M. Levine, and L. J. Noble-Haeusslein

Presenter's name: Alpa Trivedi (Mahuvakar), Ph.D.

We have shown that the matrix-metalloproteinase (MMP) inhibitor, GM6001 improves locomotor function in a murine model of moderate spinal cord contusion injury (SCI), when treatment is initiated at 3 hours post-injury. However, this timing of administration of GM6001 is not easily achievable in the clinical scenario. As infiltrating neutrophils peak at 12 hours post-injury and are a major source of MMPs, we determined if efficacy could be achieved when the timing of administration of GM6001 was extended beyond 3 hours post-injury. In this randomized and blinded study, adult male C57Bl/6 mice were subjected to a moderate-severe or a severe SCI. Animals were randomized to receive drug (GM6001 at 100 mg/kg) or vehicle (99% DMSO) starting 8 hours after injury and then every 12 hours for 3 consecutive days. Using the Basso Mouse Scale (BMS), neurological recovery was assessed at 1 and 3 days post-injury and once per week for 5 weeks. After 5 weeks, awake cystometry was conducted to assess bladder function. GM6001 treatment resulted in a long-term improvement in locomotor function and greater sparing of white matter with a corresponding reduction in lesion volume in the moderate-severe injury group. Awake cystometry revealed reduced residual urine, uninhibited bladder contractions, and bladder wall thickness relative to the vehicle control. In contrast to these beneficial effects of GM6001 seen in the moderate-severe injury group, there were no differences in neurological and urological function between GM6001 and vehicle-treated groups that sustained severe SCIs. These findings demonstrate that GM6001 shows injury-severity dependent efficacy in terms of both neurological and urological recovery. Importantly, this broad-based efficacy is achieved when the drug is administered as late as 8 hours post-injury, a feature which offers promise for the spinal cord injured patient.

Poster presentation at the American Urology Association meeting, May 16 - 21, 2014 in Orlando, Florida.

A MURINE MODEL OF SPINAL CORD INJURY: EFFECT OF THE GENERAL MATRIX METALLOPROTEINASE INHIBITOR GM6001 ON LONG-TERM LOCOMOTOR AND BLADDER FUNCTION.

Thomas M. Fandel*, Alpa Trivedi, Aida F. Martinez, Haoqian Zhang, Jonathan M. Levine, and Linda J. Noble-Haeusslein

Presenter's name: Thomas M. Fandel

Introduction and Objectives

We have previously shown that GM6001 improves locomotor function in a murine model of moderate spinal cord contusion injury (SCI), when treatment is initiated at 3 hours post-injury. Here we determine efficacy of GM6001 on locomotor and bladder function after two levels of SCI and when the initial treatment is delayed to 8 hours post-injury.

Methods

Adult, male, C57Bl/6 mice were subjected to moderate-severe (n=23) or severe (n=24) SCI, produced by dropping either a 2g or 3g weight respectively onto the exposed spinal cord at the T9 vertebral level. Injured mice were randomized to receive drug (GM6001 at 100mg/kg, n=11/12) or vehicle (99% DMSO, s.c. n=12/12) starting 8 hours after injury and then twice daily for 3 consecutive days. Nine animals served as uninjured controls. Neurological status was evaluated at 1 and 3 days post-injury and weekly thereafter for 5 weeks using the Basso Mouse Scale (BMS). At 5-6 weeks, a PE10 catheter was implanted into the bladder dome and conscious cystometry was performed 2-3 days later. Two-way repeated measures analysis of variance and unpaired T-Tests were used to evaluate locomotor recovery and bladder function, respectively. Significance was defined as $p < 0.05$.

Results

On BMS score in the moderate-severe injury group, there was an effect of both time ($p < 0.0001$) and treatment ($p = 0.04$). In contrast, in the severe injury group, there was an effect of time ($p < 0.0001$) but not of treatment on locomotor recovery ($p = 0.8$). Cystometry after moderate-severe injury revealed a therapeutic effect of GM6001 on the number of uninhibited bladder contractions (UBC)/ voiding cycle ($p = 0.007$), time to first void ($p = 0.0004$), residual urine ($p = 0.004$), and voiding efficacy ($p = 0.008$). GM6001 treatment in the severe injury group reduced the number of UBC/ voiding cycle compared to its injury control ($p = 0.03$).

Conclusions

Here we provide the first evidence that GM6001 improves both locomotor and bladder function when treatment is delayed to a more clinically relevant time point of 8 hours post-injury and after a moderate severe spinal cord injury. Such findings support the candidacy of this drug for clinical trials.

Changes in bladder function following spinal cord injury

| | Uninjured Controls | Moderate-severe SCI Controls | Moderate-severe SCI GM6001 | Severe SCI Controls | Severe SCI GM6001 |
|---|--------------------|------------------------------|----------------------------|---------------------|-------------------|
| Uninhibited bladder contractions/ voiding cycle | 1.7 \pm 0.6 | 17.2 \pm 2.7 | 8.4 \pm 1.4** | 10.6 \pm 2.0 | 4.9 \pm 1.2* |
| Time to first void (min) | 12.4 \pm 2.0 | 45.9 \pm 4.5 | 19.7 \pm 4.0*** | 30.1 \pm 6.9 | 29.5 \pm 4.4 |
| Residual urine (ml) | 0.007 \pm 0.004 | 0.648 \pm 0.047 | 0.249 \pm 0.087** | 0.463 \pm 0.088 | 0.403 \pm 0.073 |
| Voiding efficacy (%) | 93.1 \pm 3.7 | 0.2 \pm 0.1 | 35.5 \pm 10.7** | 18.6 \pm 8.6 | 20.9 \pm 11.4 |

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. respective injury controls

Published abstract: The Journal of Urology, Vol. 191, No. 45, Supplement, May 16, 2014

Poster presentation at the National Neurotrauma Society symposium, June 29-July 2, 2014 in San Francisco, CA.

MATRIX METALLOPROTEINASES AS A THERAPEUTIC TARGET FOR SUPPORTING UROLOGIC RECOVERY IN A MURINE MODEL OF SPINAL CORD INJURY

Fandel, T.M., Trivedi, A., Martinez, A.F., Zhang, H., Levine, J.M., Noble-Haeusslein, L.J.

Presenter's name: Thomas M. Fandel

Matrix-metalloproteinases (MMPs), and in particular MMP-9, are upregulated in the acutely injured spinal cord and their transient, short-term blockade with a general MMP-inhibitor (MMPI), beginning 3 hours post-injury and for the next 3 days, results in long-term locomotor recovery and greater sparing of white matter. As sparing of white matter may at least in part reflect preservation of long descending fiber tracts including those involved in the control of bladder function, we hypothesized that acute blockade of MMPs would lead to improved urological function. Testing this hypothesis, we conducted a randomized, blinded pre-clinical study, in which adult male C57Bl/6 mice were subjected to a moderate contusion injury (n=23) at the level T9 and were treated with either an MMPI or vehicle. As neutrophils are a major source of MMP-9, treatments were initiated 8 hours after injury, a time corresponding to prominent neutrophilia in the humoral compartment. Neurological and urological recovery was assessed using the Basso Mouse Scale and conscious cystometry, over a period of 5 weeks and at 6 weeks post-injury, respectively. Stereology was used to determine lesion volume and white matter sparing. As bladder dysfunction is associated with aberrant wound healing resulting in increased bladder wall thickness, this parameter was measured at the time of euthanasia. In the MMPI-treated group there were significant long-term improvements in locomotor function, sparing of white matter and voiding function, as evidenced by decreased post-void residual urine and enhanced voiding efficacy. Moreover, there were fewer uninhibited bladder contractions per voiding cycle, an indicator of decreased bladder over-activity, and detrusor wall thickness was significantly less compared to vehicle controls. In summary, delayed treatment with an MMPI improved both locomotor and bladder function. These findings, together with an extended therapeutic window, offer promise for translation to the clinical setting.

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Keywords: Spinal cord injury, Neurogenic bladder dysfunction

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SUPPORTING DATA: UCSF

Figure 1: Production of a graded reproducible model of spinal cord injury in the mouse. Two-way repeated measures ANOVA showed significant interaction ($p<0.0001$), significant effect of time ($p<0.0001$) and significant effect of injury severity ($p=0.0002$).

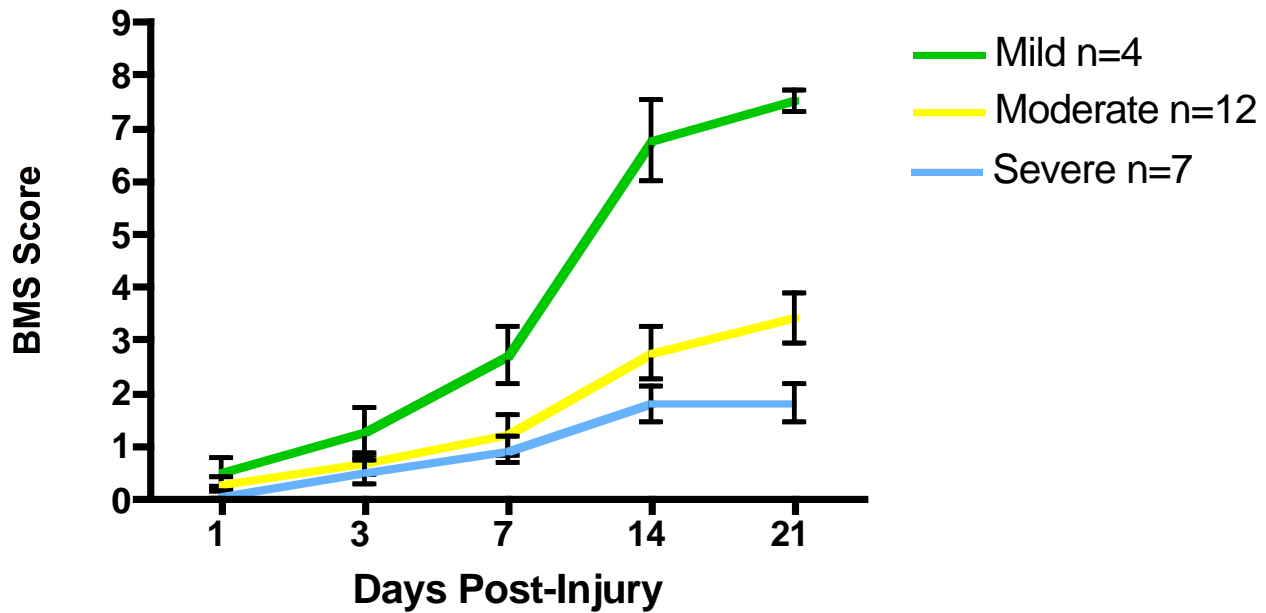
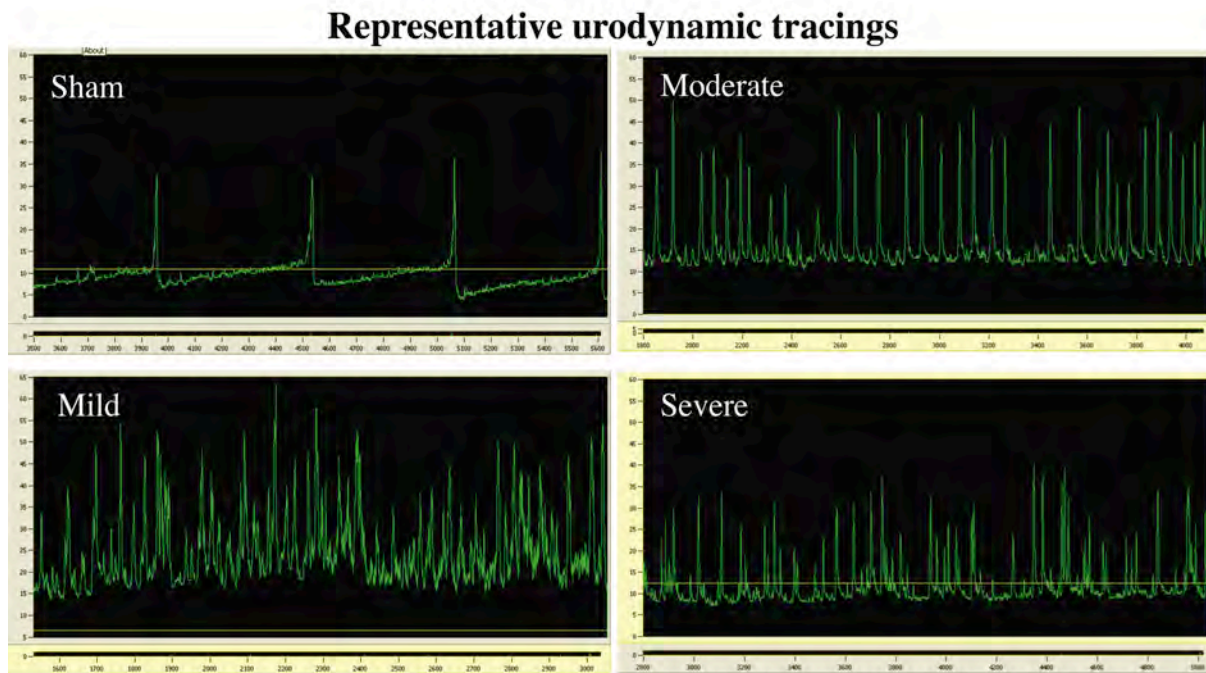


Figure 2: Uninhibited bladder contractions after mild, moderate, or severe spinal cord injury. Note the relative abundance of these contractions after mild injury relative to the more severely injured mice.



Cystometries were performed using restrained mice and a PE10 catheter with an infusion speed of 10 microliters/ minute.

Figure 3: Top Panel: Quantification of uninhibited bladder contractions after a mild and moderate spinal cord injury. Kruskal–Wallis test followed by Dunn’s Multiple Comparison Test, there were significantly increased numbers of bladder contractions after mild or moderate injury relative to the sham control group. **Bottom Panel:** Quantification of residual urine. Kruskal–Wallis test followed by Dunn’s Multiple Comparison Test, moderate injury severity had more residual urine as compared to shams.

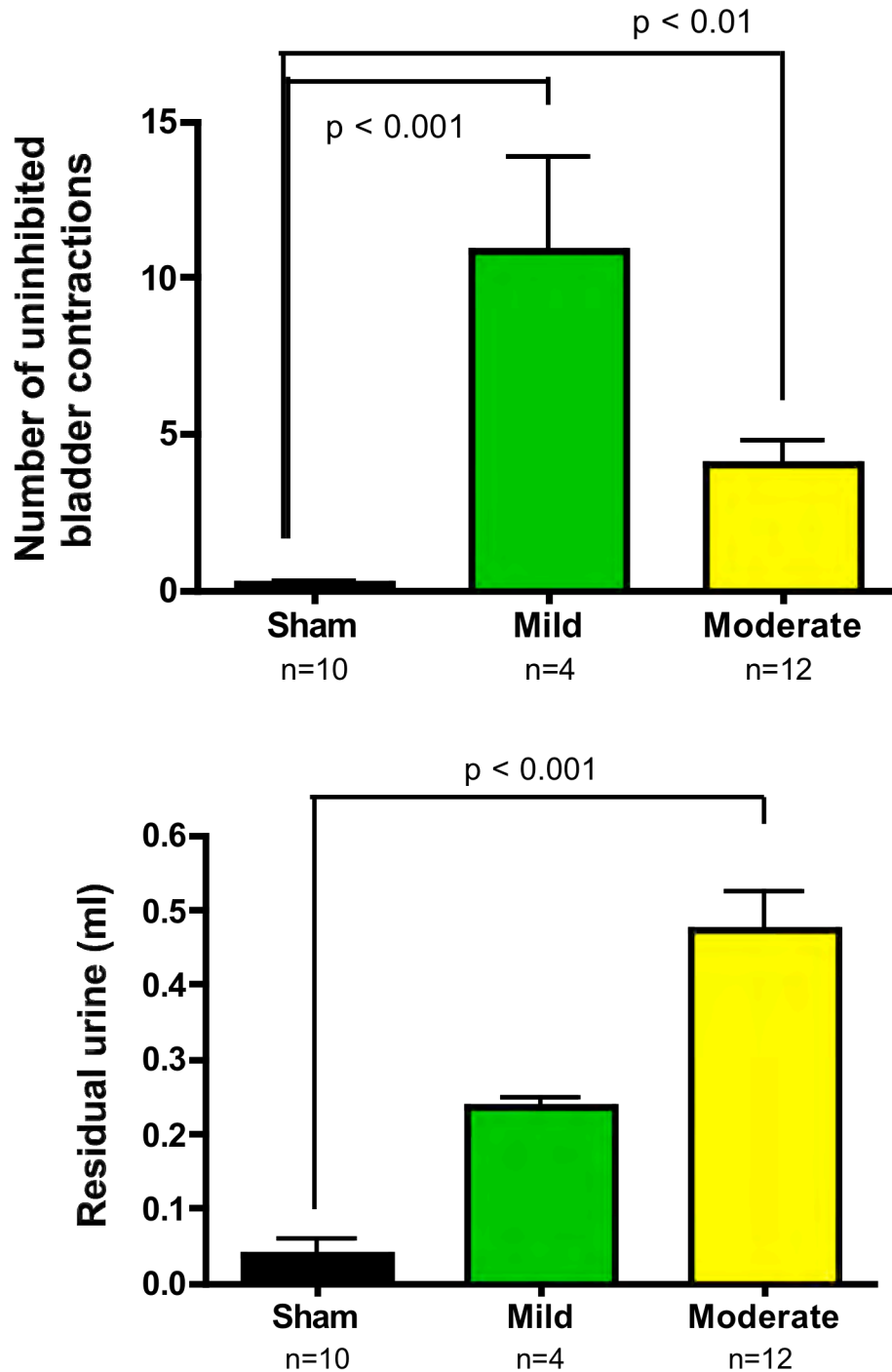


Figure 4: Peak pressure revealed no differences between groups (Kruskal–Wallis test, $p>0.05$).

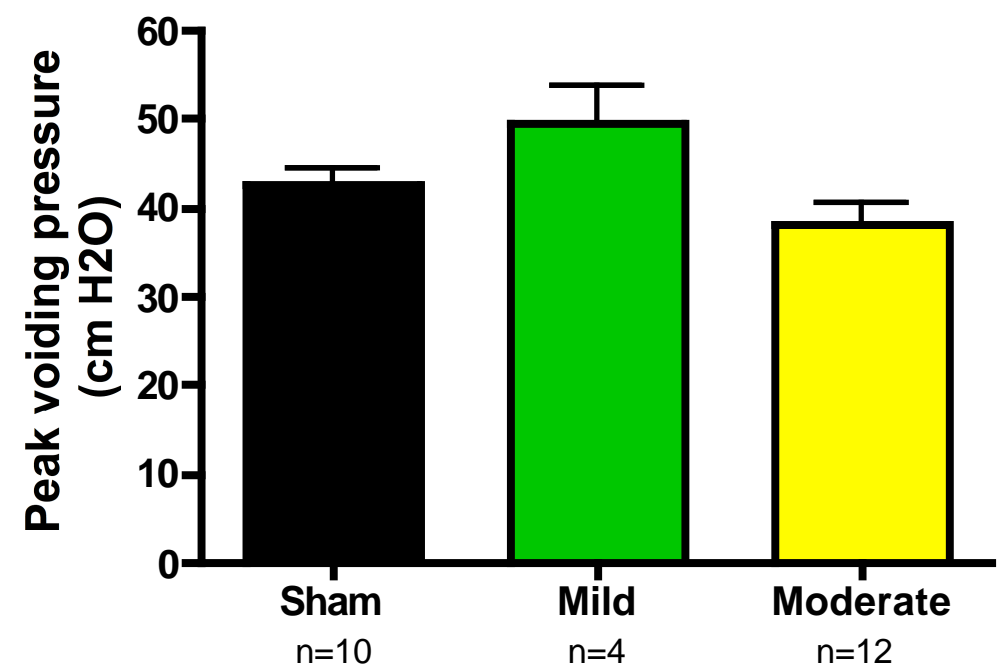


Figure 5: Bladder volume significantly increased after a moderate injury whereas there are no differences between mild and sham controls. Kruskal–Wallis test followed by Dunn’s Multiple Comparison Test, ***P<0.001

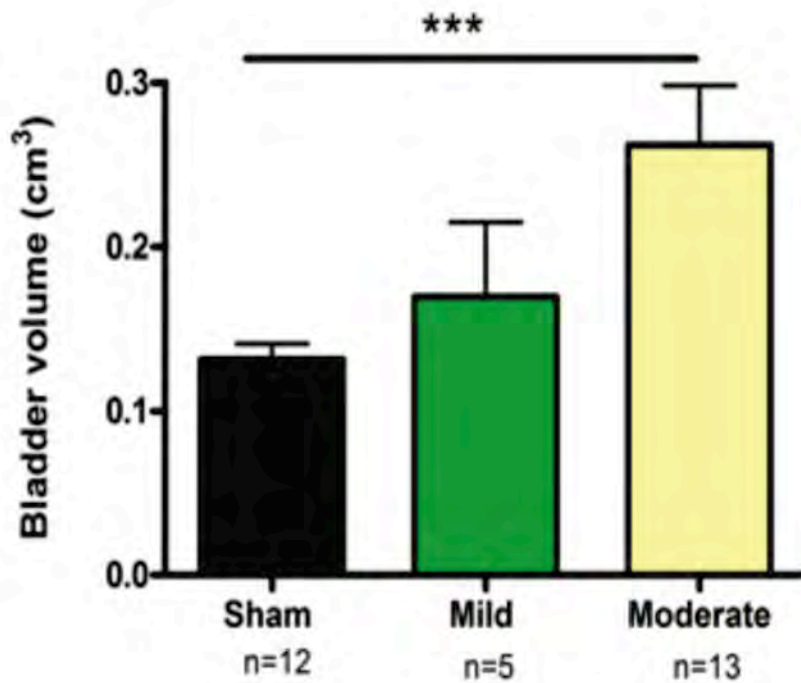


Figure 6: Bladder weight incrementally increases with injury severity. Kruskal–Wallis test followed by Dunn's Multiple Comparison Test, ***P<0.001, *P<0.05

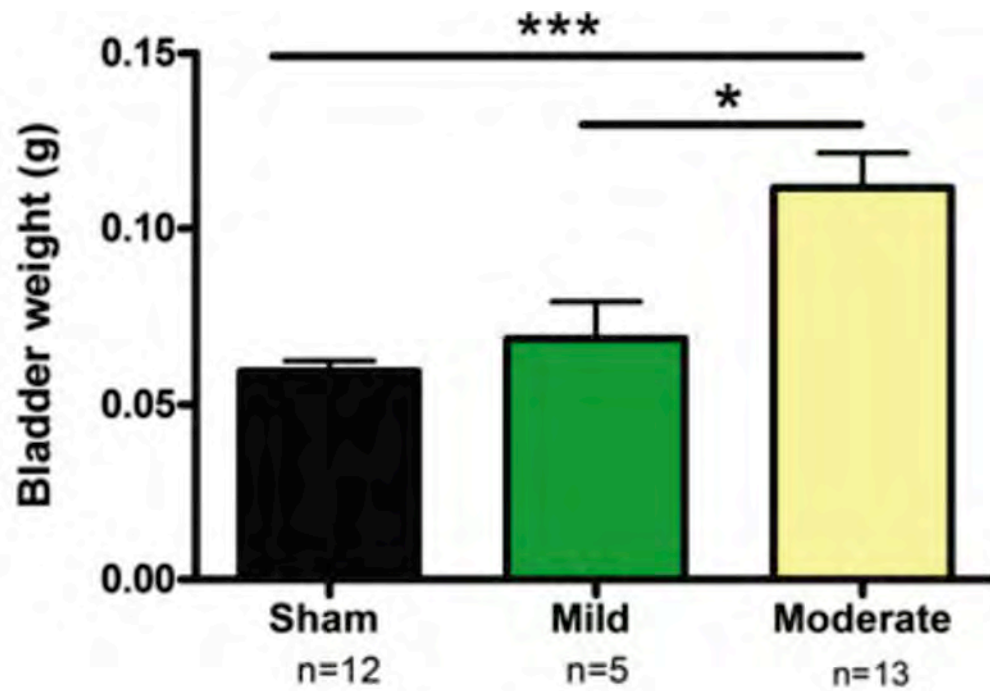


Figure 7: Male C57Bl/6 mice were subjected to a moderate spinal cord injury and treated with either GM6001 or 4% carboxy methyl cellulose beginning 8 hours post injury and every 12 hours thereafter for 3 days (route of delivery, intraperitoneal). While both groups improved locomotor function with time, there were no differences between drug and vehicle, based upon the BMS score. Two-way repeated measures ANOVA of BMS score revealed the following: $P = 0.58$ for interaction, $P < 0.0001$ for time, and $P = 0.16$ for treatment.

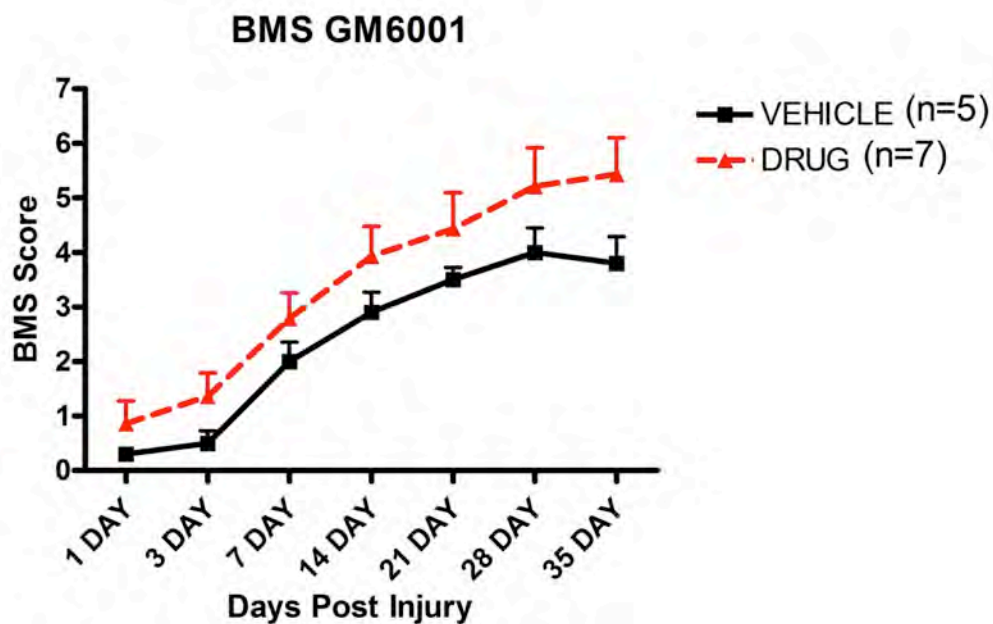


Figure 8: Comparison of initial BMS scores at 1 day versus 35 days, revealed a significant improvement in the GM6001-treated group. Student T-test, * P=0.025

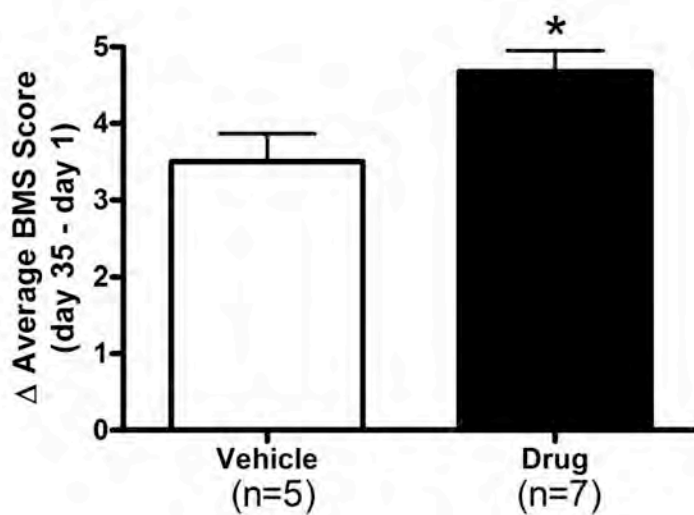


Figure 9: Evaluation of percentage of mice, subjected to a moderate spinal cord injury and GM6001 treatment show hindlimb stepping over time. Two way ANOVA, no significant interaction, significant effect of both treatment ($P= 0.017$) and time ($P= 0.015$).

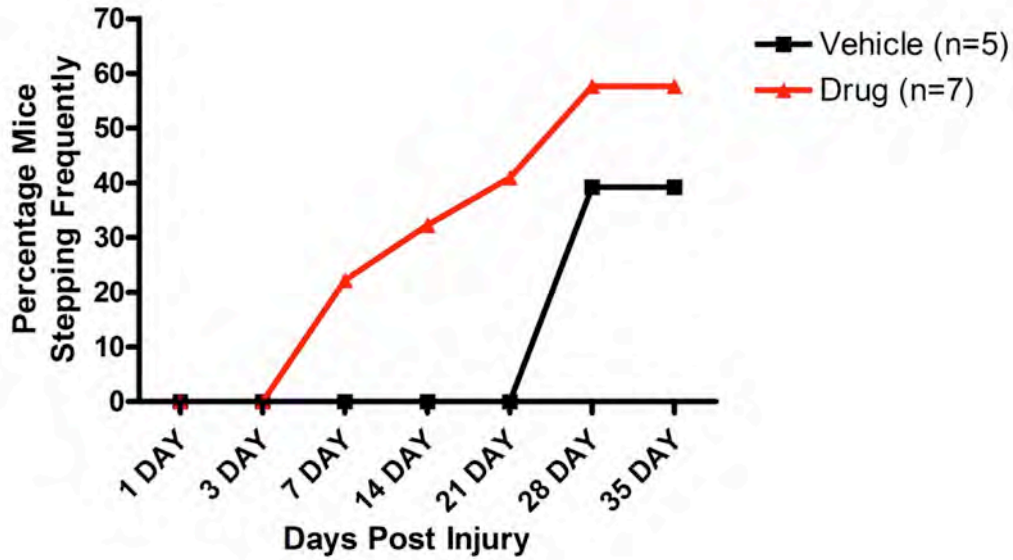


Figure 10: Analysis of bladder function after drug treatment in moderate spinal cord injury in mice. Cystometry was conducted in those animals that were also evaluated for neurologic function (Refer to Figures 7-9). There were signature reductions in number of uninhibited bladder contractions (Unpaired Students T-test).

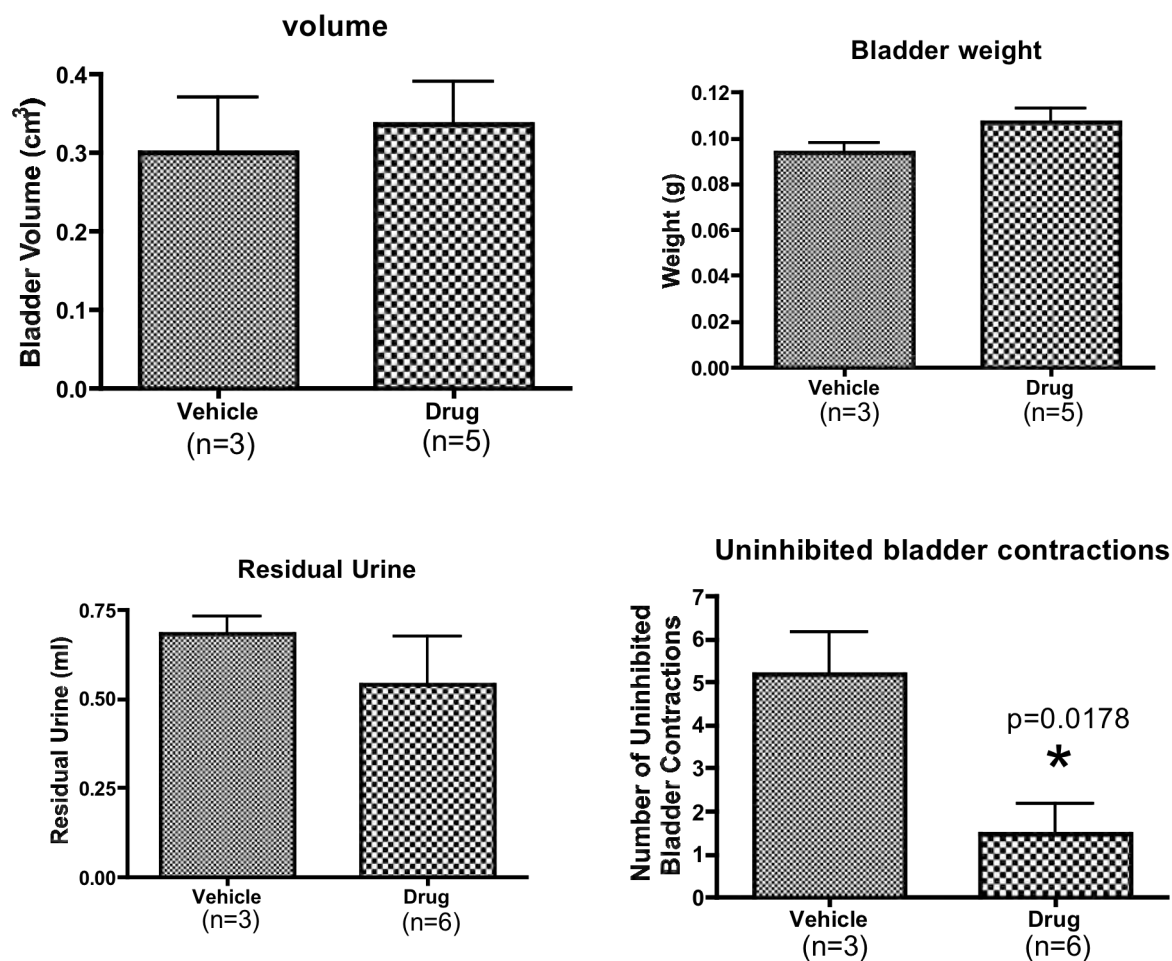


Figure 11: Number of mice used for neurologic and urologic behavior testing and for immunohistochemistry. *We had set a priori exclusion criteria: any animal showing extensive ankle movement of either one or both hind limbs (average score >0.5) at eight hours post-injury would be removed from the study. Seven mice fell into this category. Two additional animals with adverse events were removed from the study, one due to weight loss and another one due to self-mutilation. In the drug treatment group, one mouse did not undergo cystometry for technical reasons, but was included in all other outcome measures. Abbreviations: SCI (spinal cord injury), BMS (Basso Mouse Scale), and SC (spinal cord).

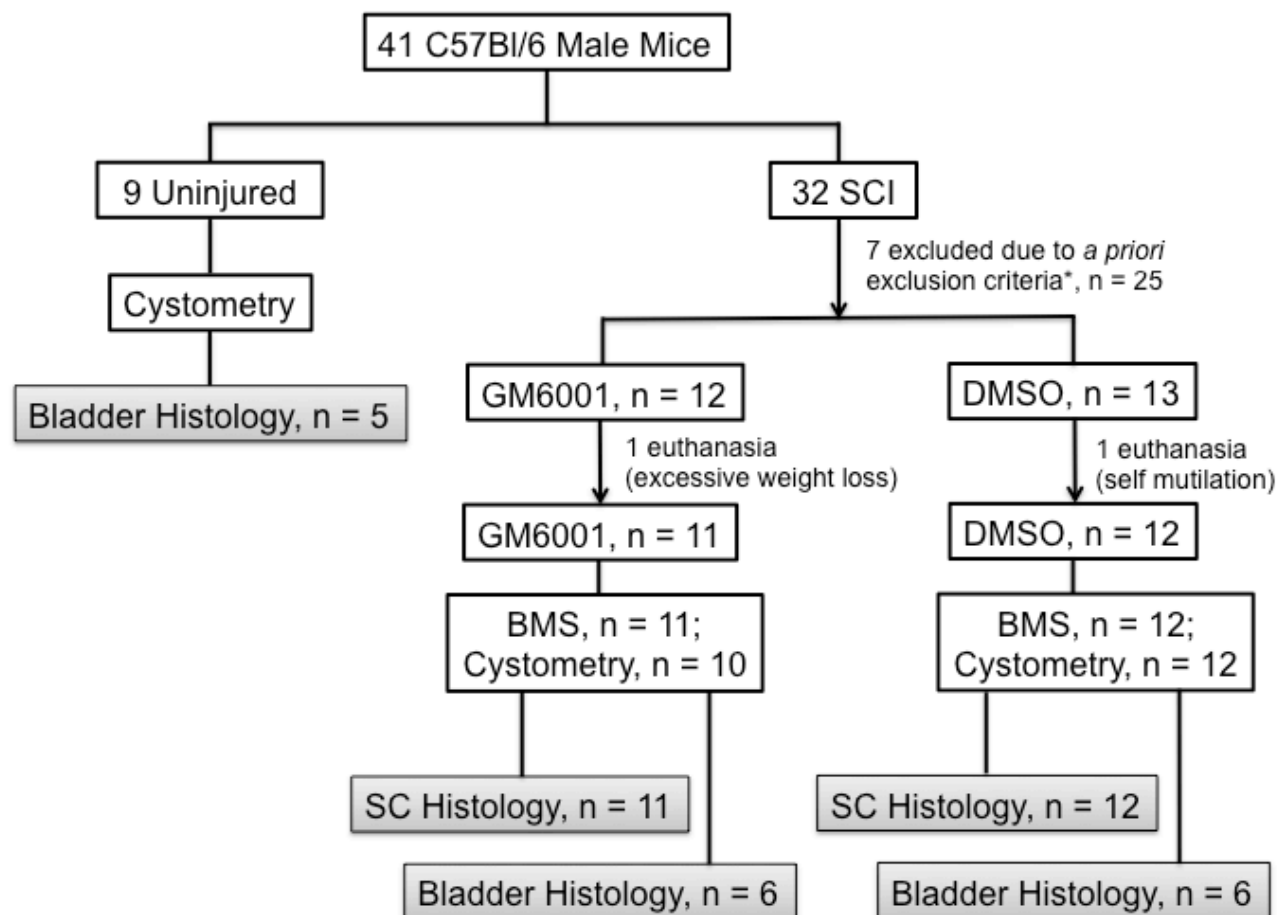


Figure 12: Delayed GM6001 treatment improves locomotor function after moderate SCI. **A**, Basso mouse scale (BMS) score shows that both groups improve locomotor function over time; nonetheless, GM6001 treatment results in improved locomotor function as compared to vehicle treated group [effect of treatment ($p=0.0397$)]. At 35 days post-injury, vehicle treated mice on an average had a score of 3.3 (ability to plantar place but no plantar stepping). The GM6001 treated group had an average score of 4.3 (mice display occasional plantar stepping). Values are mean + SEM. **B**, Higher percentage of GM6001-treated mice had the ability to step as compared to vehicle-treated mice, chi square analysis, $*p=0.0294$, DMSO: $n = 12$; GM6001: $n = 11$.

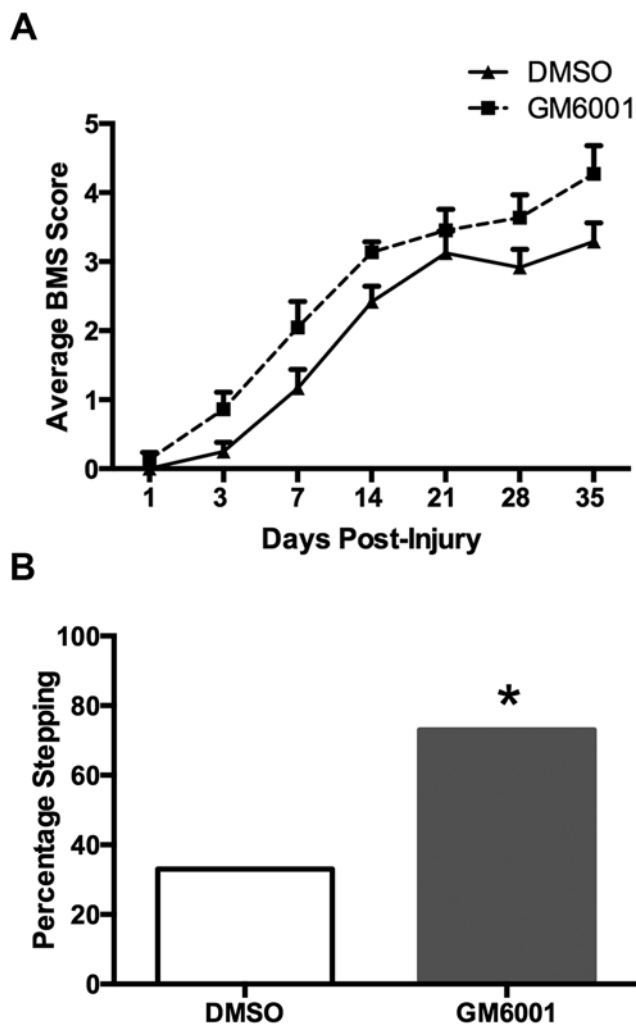


Figure 13: Male C57Bl/6 mice were subjected to a moderate spinal cord injury and treated with either GM6001 or DMSO (vehicle), injected subcutaneously, beginning 8 hours post injury and every 12 hours thereafter for 3 days. Repeated measures Two-way ANOVA of percentage weight change revealed the following: $P = 0.0317$ for interaction, followed by Sidak's multiple comparisons test. Within group comparisons showed that GM6001 treated group did not have a significant weight loss as compared to pre-injury values, whereas, the vehicle treated group had significant weight loss over the first week. Between group comparisons showed that GM6001 treated mice showed a greater weight gain as compared to vehicle treated group ($*p < 0.05$). DMSO: $n = 12$; GM6001: $n = 11$.

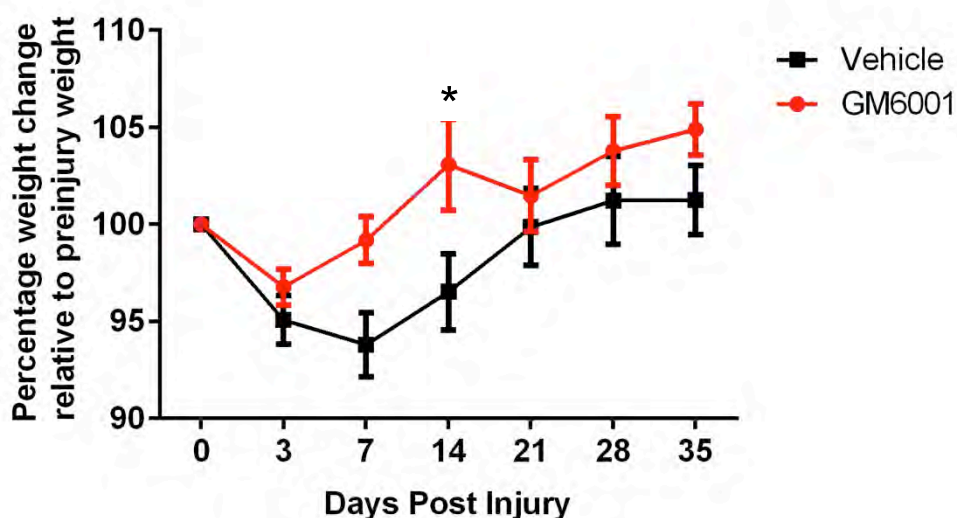


Figure 14: Male C57Bl/6 mice were subjected to a severe spinal cord injury and treated with either GM6001 or DMSO (vehicle) beginning 8 hours post injury and every 12 hours thereafter for 3 days. While both groups improved with time, there were no differences between drug and vehicle treated groups based upon BMS scale. Two-way repeated measures ANOVA of BMS score revealed the following: $P = 0.9468$ for interaction, $P < 0.0001$ for time, and $P = 0.7530$ for treatment.

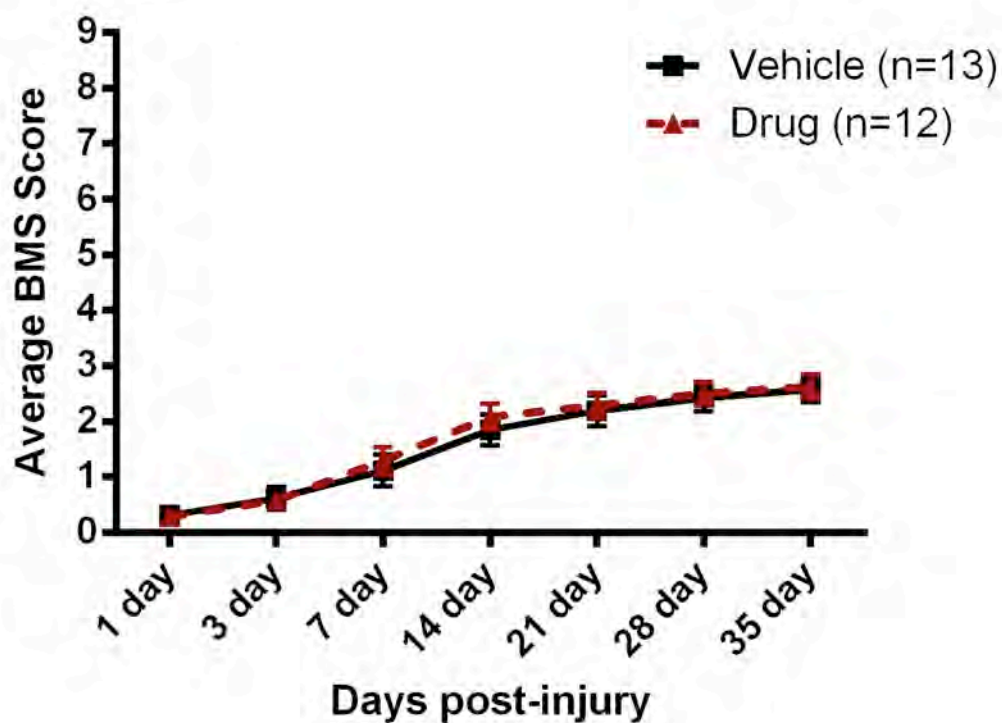


Figure 15: Male C57Bl/6 mice were subjected to a severe spinal cord injury and treated with either GM6001 or DMSO (vehicle), injected subcutaneously, beginning 8 hours post injury and every 12 hours thereafter for 3 days. Both gained weight with time but GM6001 treatment did not affect weight gain as compared to vehicle treated group. Repeated measures Two-way ANOVA of weight revealed the following: $P = 0.2662$ for interaction, $P < 0.0001$ for time, and $P = 0.5362$ for treatment.

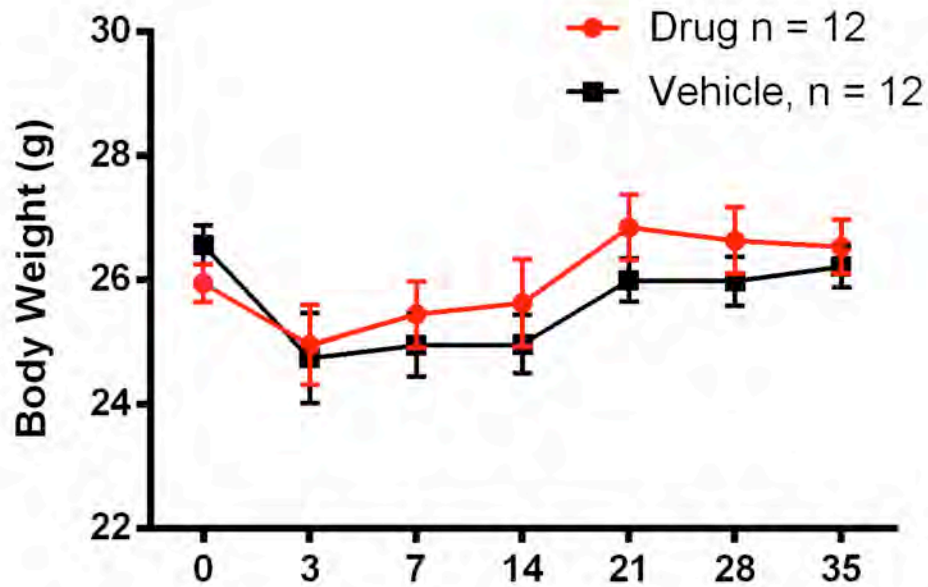


Table 1: Cystometric parameters

| | Uninjured ^a (n = 9) | SCI DMSO (n = 12) | SCI GM6001 (n = 10) |
|--|-----------------------------------|-------------------------|------------------------------|
| Baseline pressure (cm H ₂ O) | 7.51 ± 1.21 | 10.68 ± 0.70 | 7.95 ± 0.83 [#] |
| Threshold pressure (cm H ₂ O) | 18.41 ± 1.99 ^{##} | 13.40 ± 1.24 | 13.32 ± 0.92 |
| Opening pressure (cm H ₂ O) | 30.07 ± 1.82 | 24.97 ± 2.75 | 24.51 ± 2.15 |
| Maximum pressure (cm H ₂ O) | 49.46 ± 2.93 ^{###} | 34.38 ± 2.60 | 34.14 ± 1.96 |
| Δ bladder pressure (cm H ₂ O) | 41.94 ± 2.57 ^{###} | 23.70 ± 2.13 | 26.19 ± 2.43 |
| Voiding duration (s) | 31.19 ± 2.57 [*] | 44.31 ± 3.50 | 38.44 ± 2.93 |
| NVCs/ voiding cycle | 1.07 ± 0.24 ^{####} | 15.04 ± 2.46 | 7.32 ± 0.99 ^{##} |
| Δ NVCs increase (cm H ₂ O) | 6.23 ± 0.35 ^{****} | 12.58 ± 0.58 | 10.56 ± 0.66 [*] |
| Time to first voiding/leak (min) | 15.83 ± 2.27 ^{****} | 38.19 ± 3.20 | 24.14 ± 3.84 [*] |
| Intermicturition interval (min) | 17.94 ± 2.38 | 13.48 ± 1.29 | 13.86 ± 2.35 |
| Residual urine (ml) | 0.006 ± 0.004 ^{****} | 0.643 ± 0.047 | 0.250 ± 0.086 ^{***} |
| Voiding efficiency (%) | 92.9 ± 4.1 ^{####} | 0.2 ± 0.1 | 37.5 ± 12.8 ^{##} |

^a Uninjured animals served to establish baseline values. Data for all groups shown as mean ± SEM. Δ bladder pressure is the difference between baseline pressure and maximum pressure. NVCs: non-voiding contractions.

*/[#]:p<0.05, ^{##}:p<0.01, ^{***}/^{###}:p<0.001, ^{****}/^{####}:p<0.0001 vs. DMSO treatment

Asterisks denote unpaired t tests, while hash-tags denote Mann-Whitney tests.

Figure 16: GM6001 treatment improves urodynamic measures after moderate SCI. **A – D**, Cystometry results demonstrate that drug treatment decreases the development of large bladder capacities, the emergence of non-voiding bladder contractions per micturition cycle, and the retention of urine. Overall, voiding efficiency is improved after drug treatment. Black line indicates the baseline value for uninjured animals. Values are mean + SEM; DMSO: n = 12; GM6001: n = 10, Uninjured: n = 9 (**p<0.01, ***p<0.001). **A, C**: unpaired t tests; **B, D**: Mann-Whitney tests. **E – G**, Representative voiding and bladder pressure recordings (+ indicates voiding/ urine leakage) were recorded for each group at 6 weeks post-injury. Drug treatment resulted in fewer non-voiding bladder contractions per micturition cycle and a better voiding efficacy, signified by the return of pressure to baseline values after voiding.

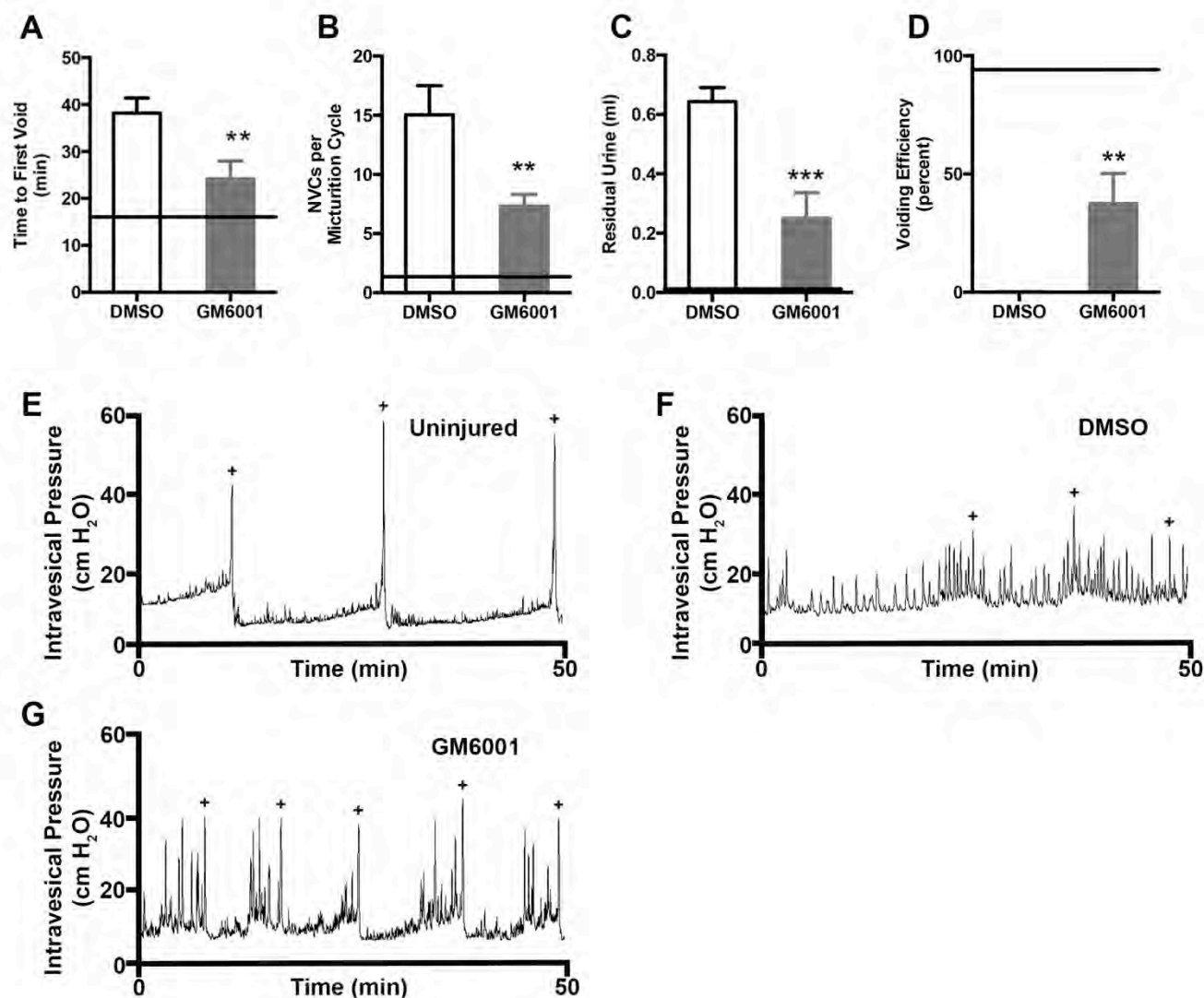


Figure 17: GM6001 treatment decreases bladder weight and bladder wall thickness after moderate SCI. The bladder increases in size in response to injury, as depicted by weight and wall thickness. **A**, Drug treatment does not significantly reduce bladder weight; however, there is a strong trend of reduced bladder weight in the drug-treated group as compared to the vehicle-treated group (Mann-Whitney test: $p = 0.0509$); DMSO: $n = 12$; GM6001: $n = 11$. **B**, There is a significant reduction in wall thickness in each separate bladder region in the drug-treated group as compared to the vehicle-treated group (unpaired t tests: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Black line indicates the baseline value for uninjured animals. Values are mean \pm SEM; DMSO: $n = 6$; GM6001: $n = 6$; Uninjured: $n = 5$. **C – H**, H&E staining of representative bladder sections. The muscle layer is shown in pink. Note the decreased detrusor muscle thickness in each bladder region following drug treatment as compared to vehicle treatment.

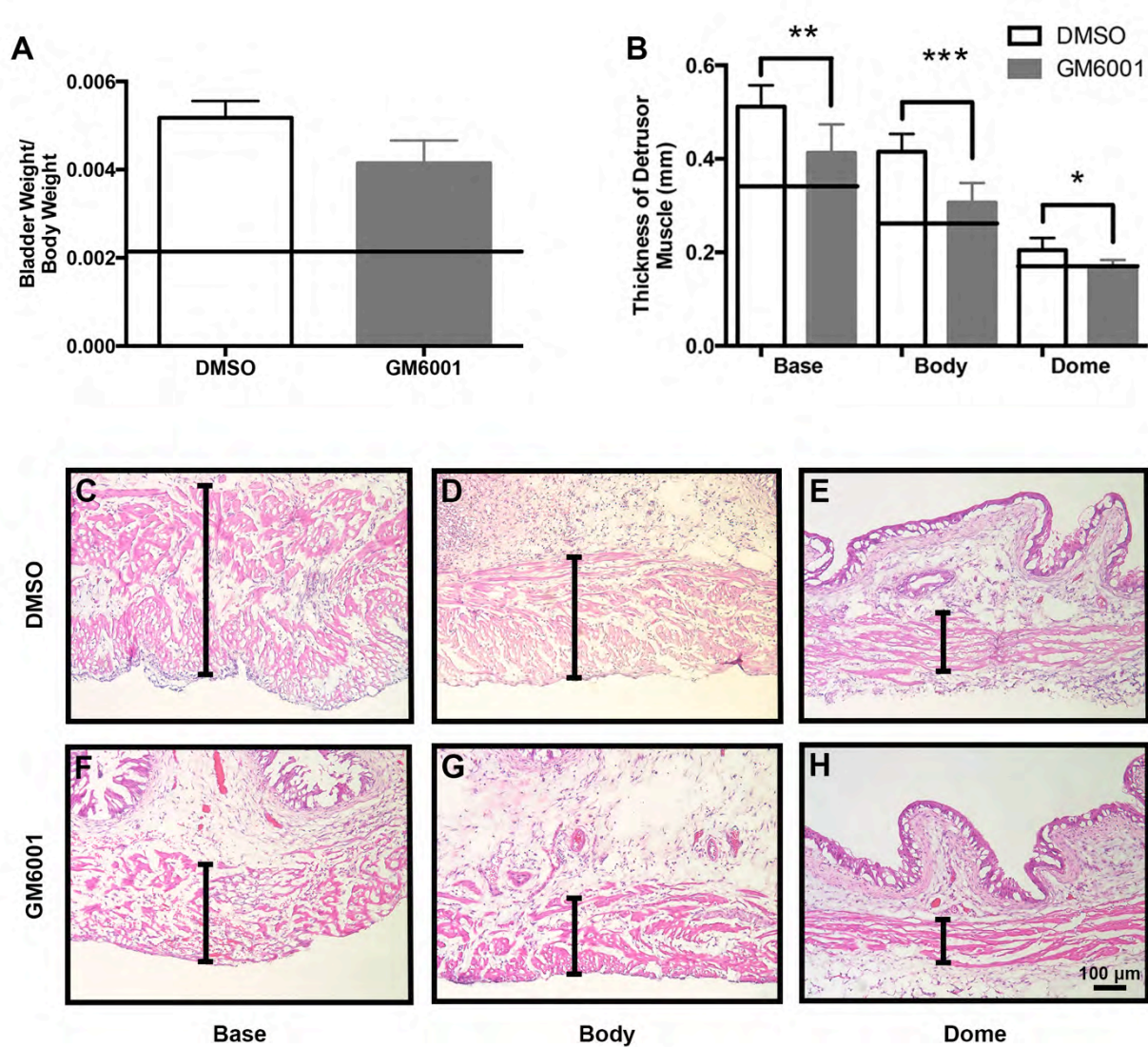


Figure 18: GM6001 treatment does not affect collagen III/collagen I ratio within the detrusor muscle. Morphological changes in bladder wall at six weeks post-injury reveal **A**, injury results in a reduction in collagen III/collagen I ratio, and this is not rescued by GM6001 treatment. **B-M**, representative images from the body region of the bladders of uninjured (**B-E**), vehicle-treated (**F-I**) and GM6001-treated (**J-M**) mice. Bars represent mean + SEM; DMSO: n = 6; GM6001: n = 6; Uninjured: n = 5. Scale bar in M denotes 100 μ m.

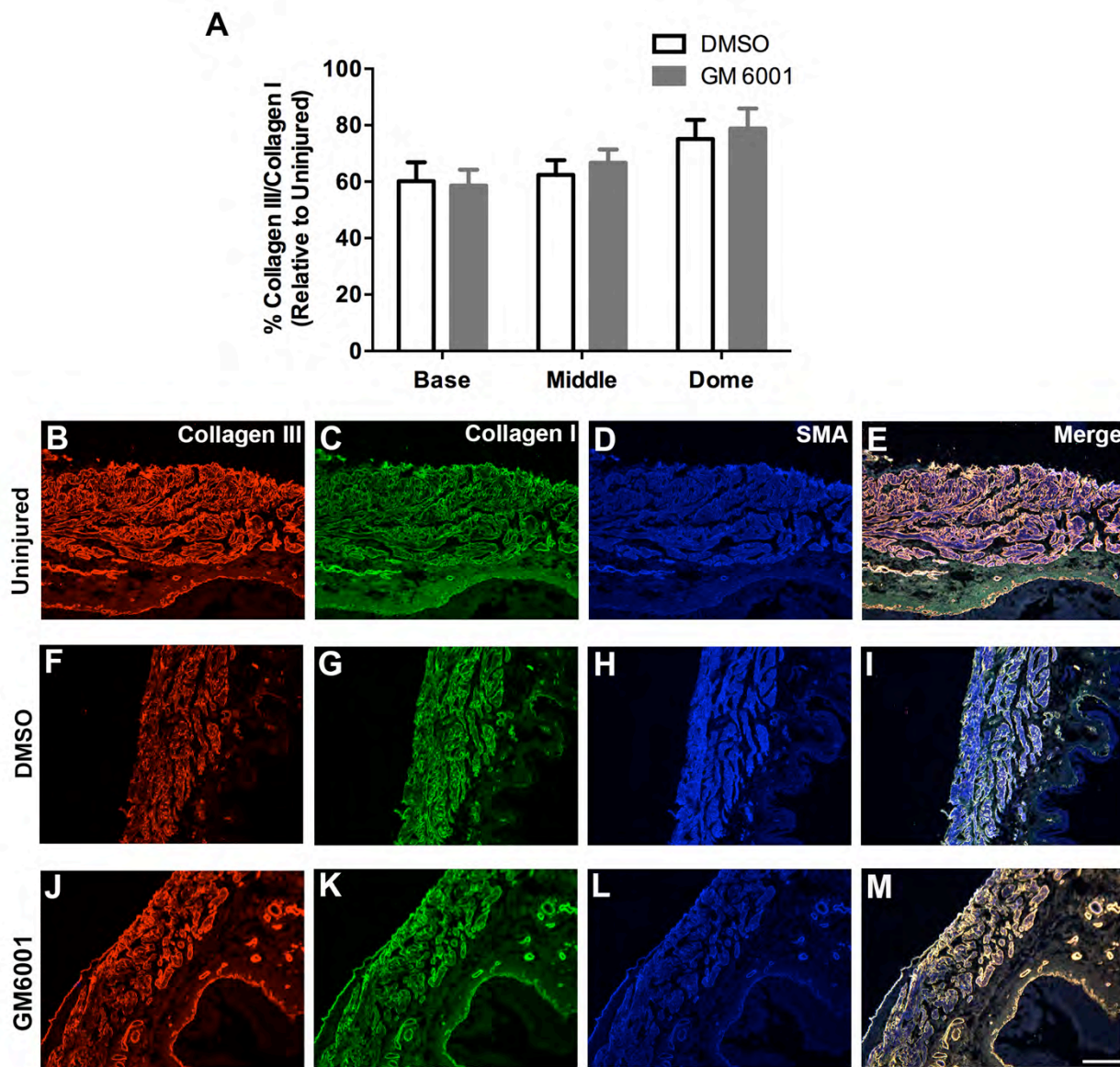


Figure 19: *In situ* gelatinolytic activity in the bladder in response to spinal cord injury. Unfixed bladders from mice (uninjured or at 7 days after injury) were frozen, and cryosections were prepared for *in situ* gelatin zymography. Fluorescence is indicative of gelatinolytic activity. In the uninjured bladders, small amount of cellular gelatinase activity is identified within the urothelium. Seven days after spinal cord injury, gelatinase activity is prominent within the bladder urothelium.

In Situ Zymography

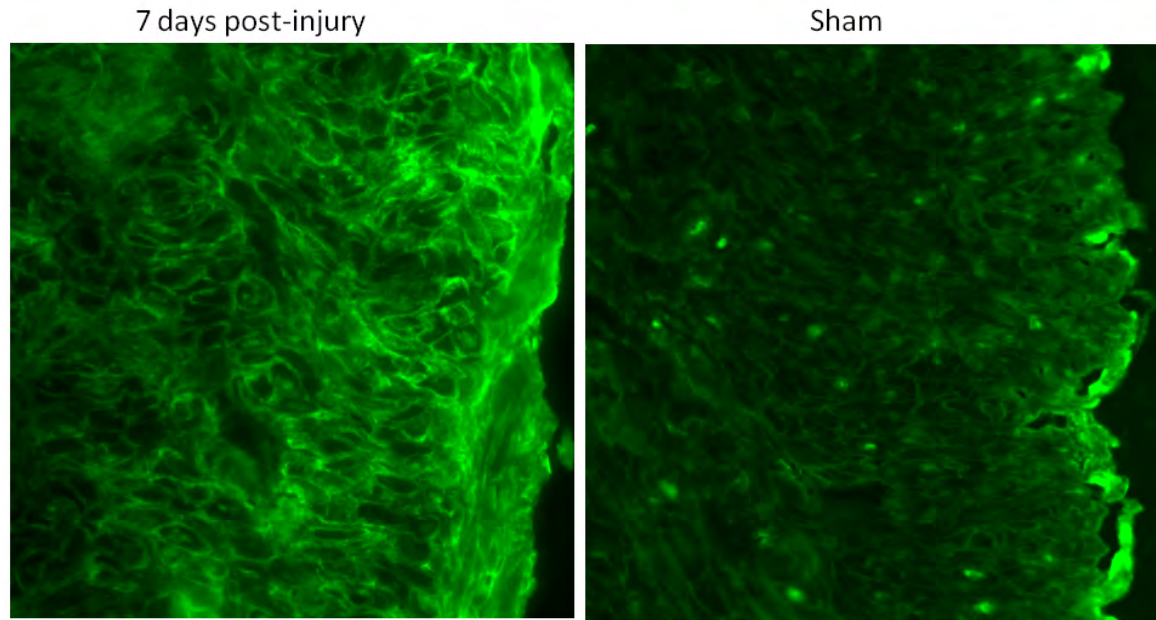


Figure 20: Urodynamic outcomes from awake cystometries of severely injured mice treated with GM6001 or vehicle. Drug treatment did not affect urine retention (Unpaired two-tailed T- test, $p=0.6038$), decreased number of uninhibited bladder contractions per cycle (Unpaired two-tailed T- test, $p=0.0210$), but had no effect on time to first void (Unpaired two-tailed T- test, $p=0.9573$), or voiding efficacy (Mann Whitney test, $p=0.4401$). Red lines in each graph indicate baseline values of uninjured male C57Bl/6 mice. Bars represent mean \pm SEM.

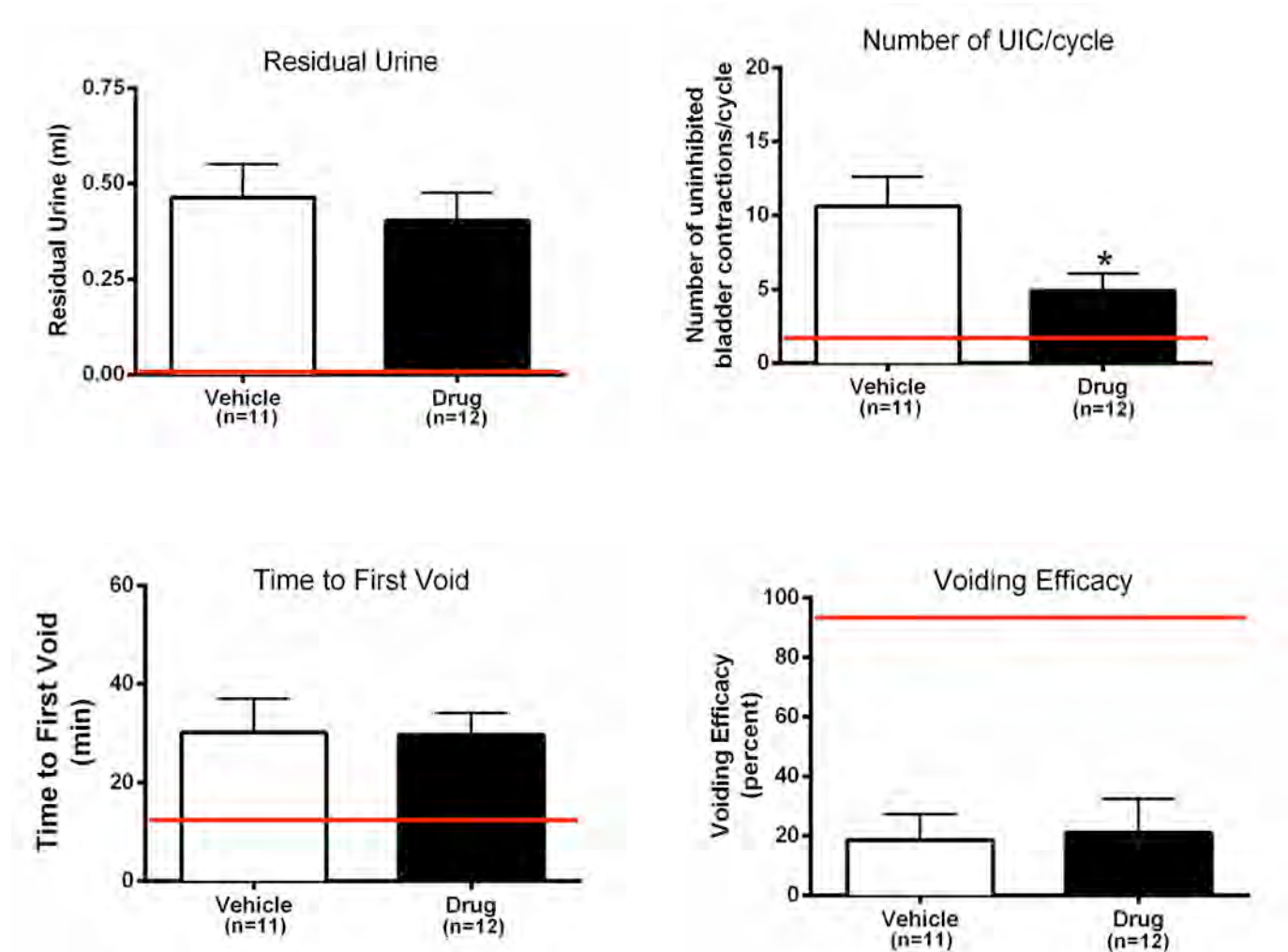


Figure 21: Bladder weights were analyzed from severely injured mice, treated with either vehicle or drug. Drug treatment did not lead to significant decrease in bladder weight (Unpaired two-tailed T test, $p=0.1390$). Bars represent mean+SEM.

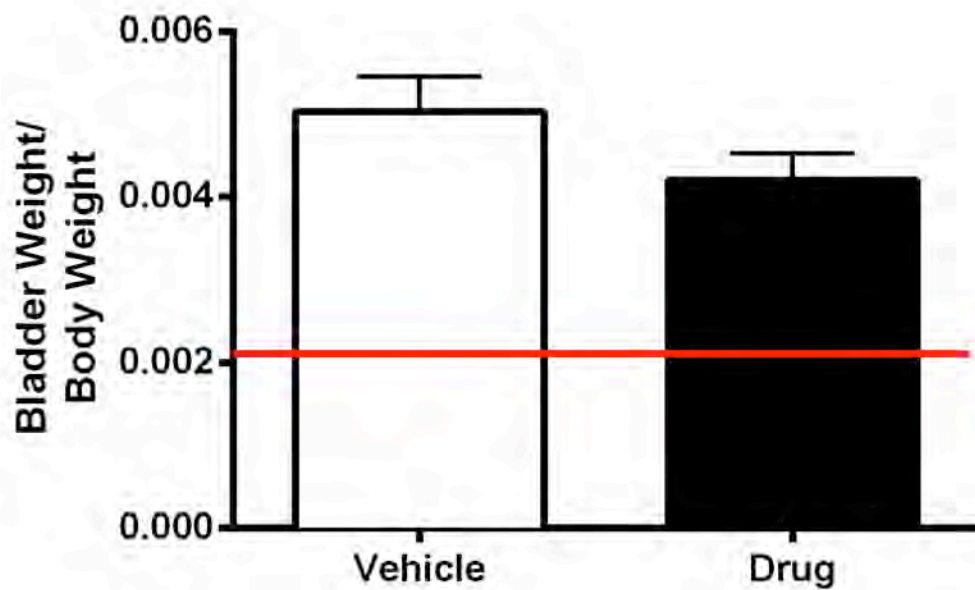


Figure 22: Delayed treatment with GM6001 reduces lesion volume and increases white matter sparing in moderately injured mice. **A**, In both groups, lesion volume decreases over a distance of 3 mm at regions removed from the lesion epicenter. Drug treatment significantly decreases lesion size at 400 and 600 μm caudal to the epicenter ($***p<0.001$). **B**, White matter sparing increases at regions removed from the lesion epicenter. Drug treatment significantly increases white matter sparing at 400 and 600 μm caudal to the epicenter ($**p<0.01$). Stereological 3D reconstruction of representative animals from the vehicle (**C**) and drug-treated (**D**) groups illustrate the differences between groups exhibited histologically. Red represents lesion, blue represents white matter and green represents gray matter. The isolated vehicle-treated injury lesion (**E**) is larger than that of an animal given GM6001 (**F**). These differences are also shown in the isolated 3D reconstruction of the gray matter from the vehicle-treated animal (**G**), which is smaller than that of an animal that received drug treatment (**H**). Values are mean \pm SEM, DMSO: $n = 12$; GM6001: $n = 11$.

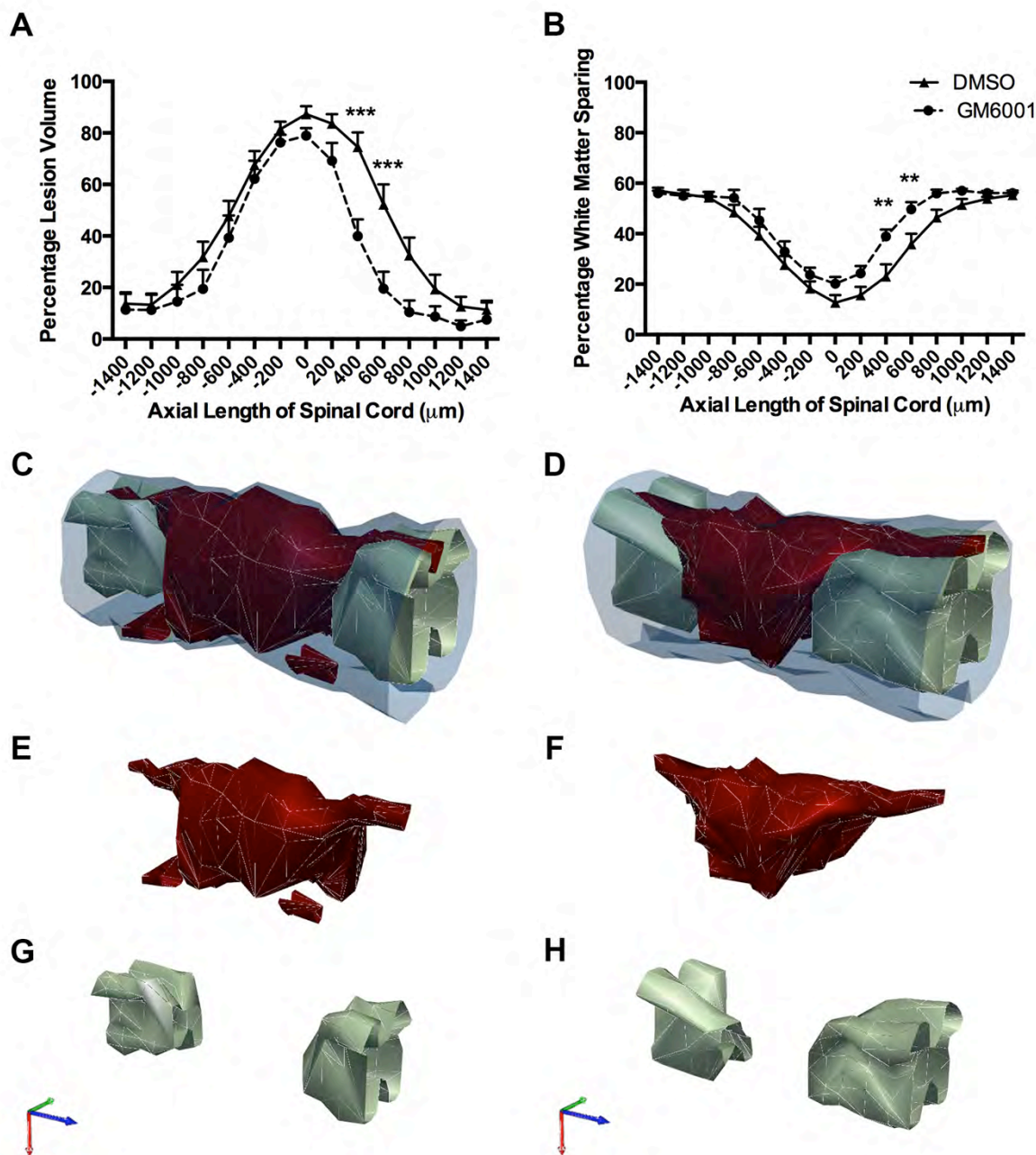


Figure 23: The rostral and caudal extent of lesion volume in severely injured mice treated with GM6001 or vehicle was quantified at 200- μ m intervals spanning from 1400 μ m rostral to 1400 μ m caudal to the epicenter. Percentage lesion decreased at distances further removed from the epicenter in both groups but no differences were found between the groups in lesion volume. Two-way ANOVA, interaction, $p=0.7712$; effect of distance, $p<0.0001$; effect of treatment, $p=0.1064$.

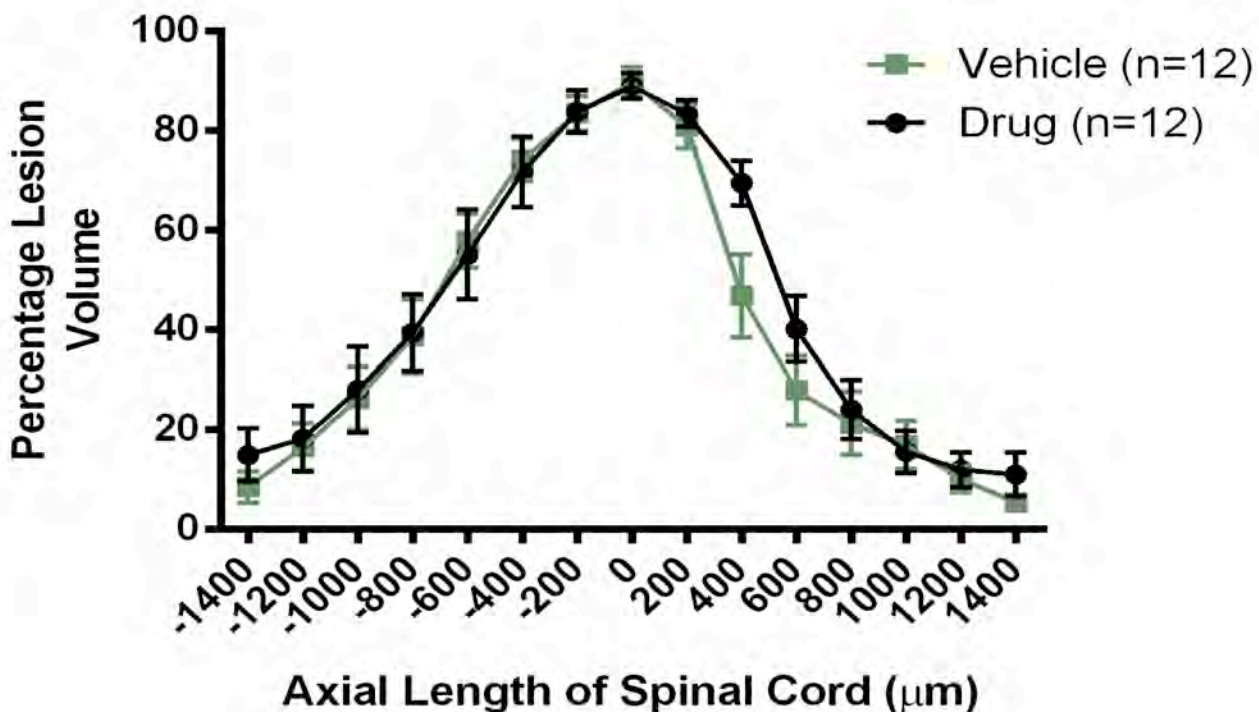
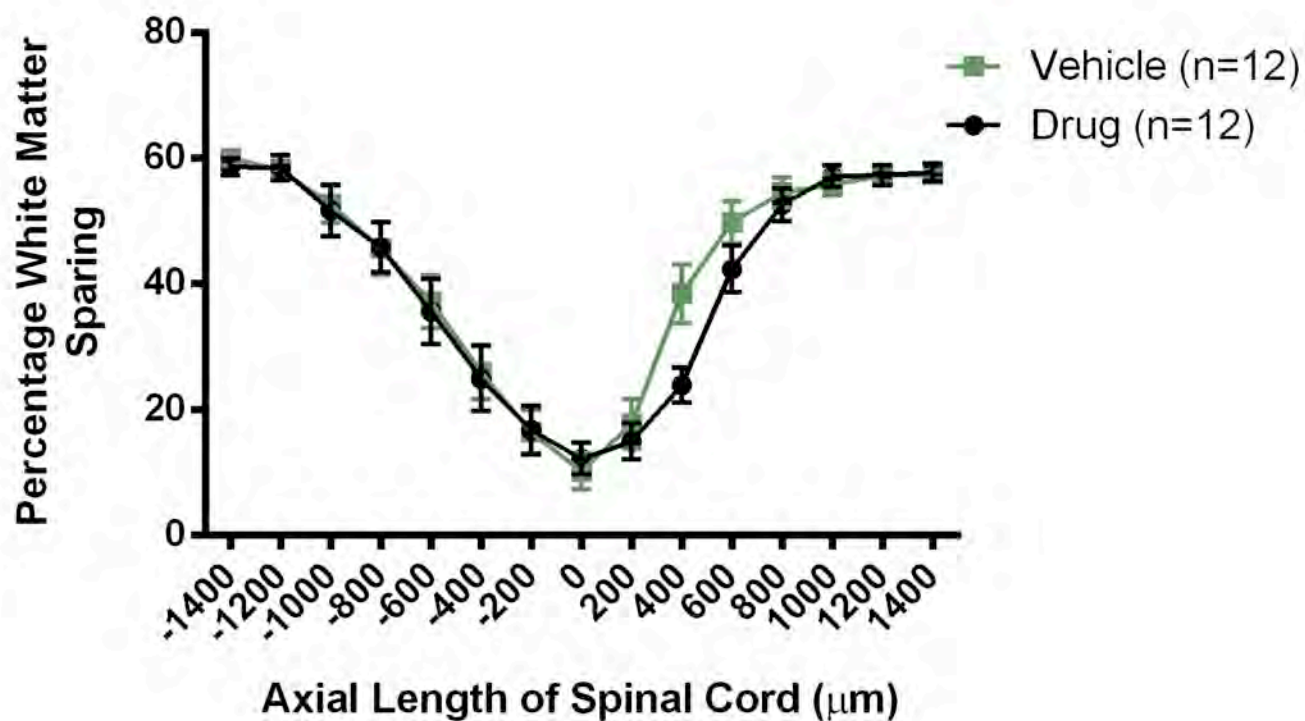


Figure 24: The rostral and caudal extent of spared white matter in severely injured mice treated with G M6001 or vehicle was quantified at 200- μm intervals spanning from 1400 μm rostral to 1400 μm caudal to the epicenter. Percentage spared white matter increased at distances further removed from the epicenter in both groups but no differences were found between the groups in spared white matter. Two-way ANOVA, interaction, $p=0.6069$; effect of distance, $p<0.0001$; effect of treatment, $p=0.1235$.



SUPPORTING DATA: - TAMU

Figure 1: Quantification of MMP and ADAM activity in normal and SCI dogs. Dogs with SCI have significantly greater MMP-9 and ADAM-7 expression compared with healthy controls. No other MMPs or ADAMs were detected in the CSF of normal or SCI dogs. Dogs with severe SCI (MFS = 0) did not have significantly greater expression of MMP-9 or ADAM-7 than those with mild-to-moderate SCI (MFS = 3)

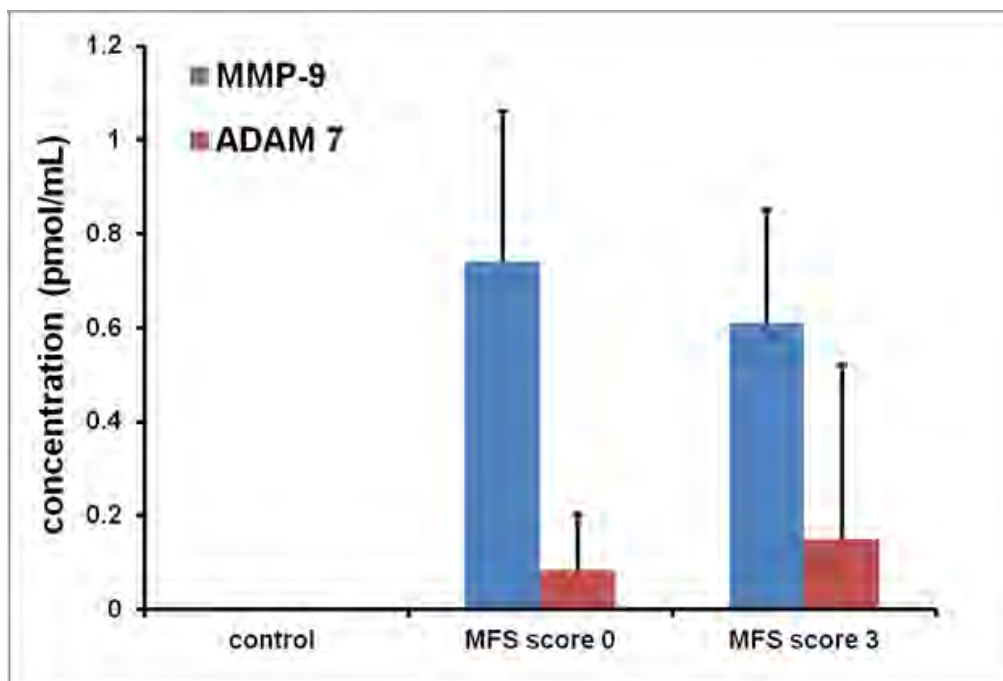


Figure 2: Plasma concentration of GM 6001 in 5 dogs dosed once at 100 mg/kg S.C. and 5 dogs dosed twice at a 12-hour interval. Data for the two-dose cohort was collected only at time points following the second dose.

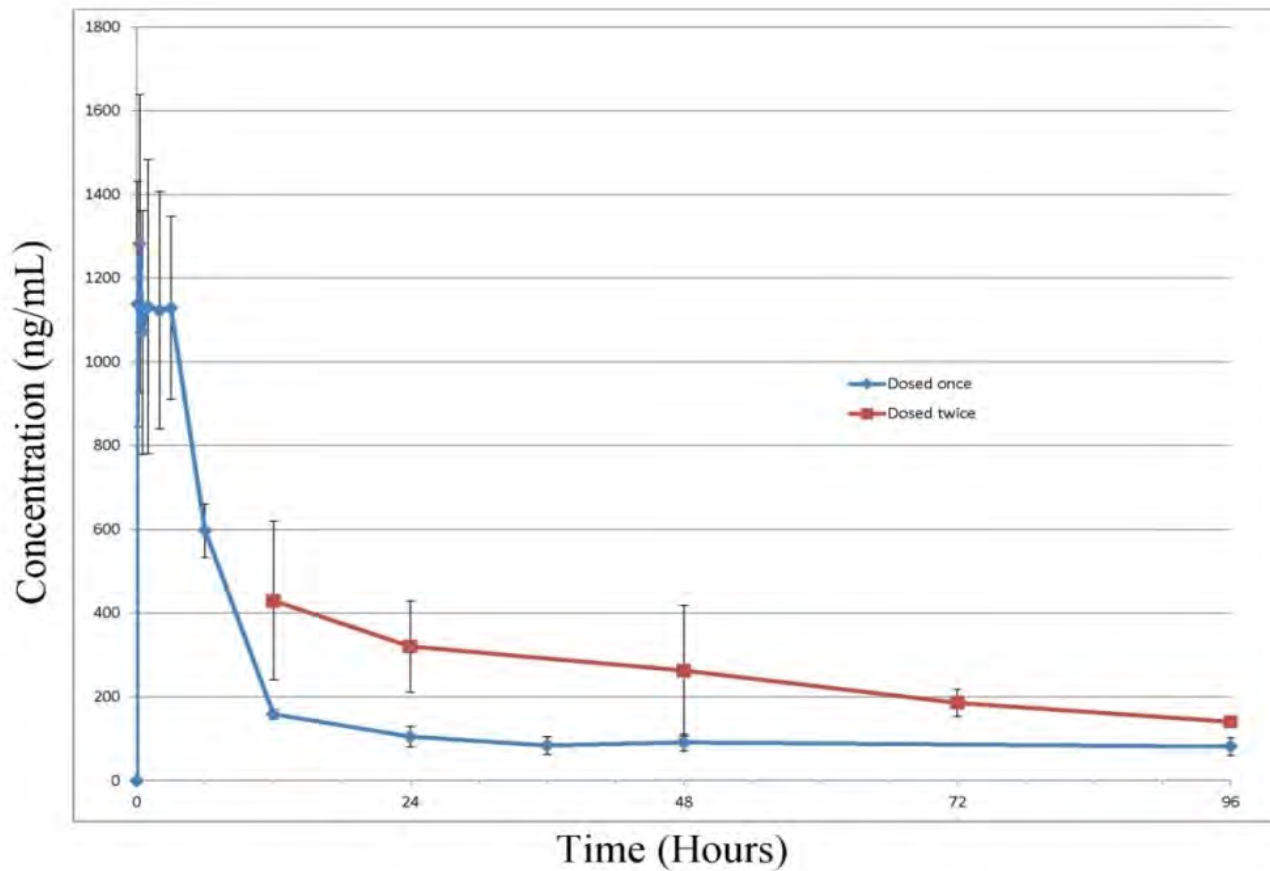


Figure 3. Cystometry 24 hours following spinal cord injury in a dog with intervertebral disk herniation. Note the absence of voiding, the presence of uninhibited bladder contractions (U), and the low leak point pressure (12 mm H₂O)

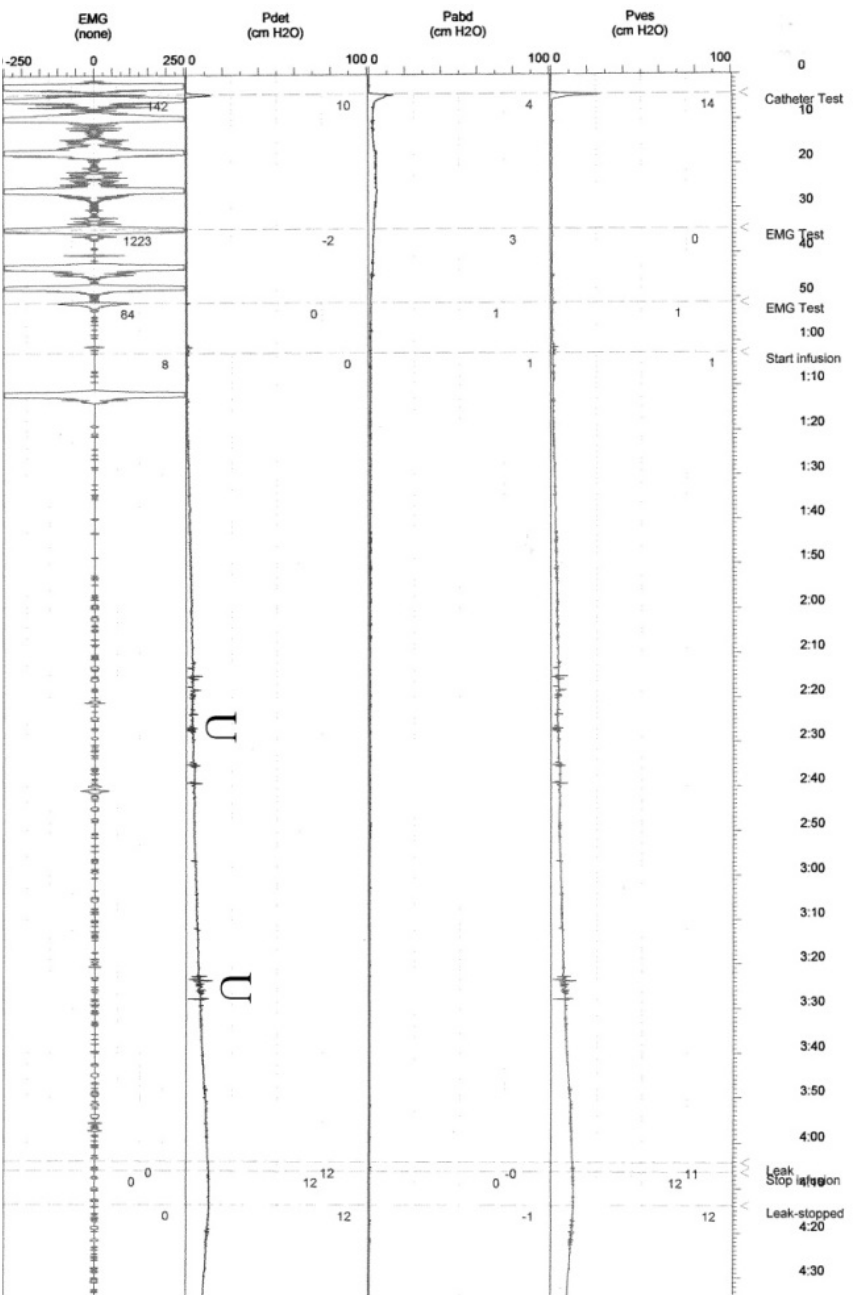


Figure 4. Cystometry 3 days following Figure 2. Note the presence of a voiding reflex, the absence of un-inhibited bladder contractions, and the presence of anal EMG activity.

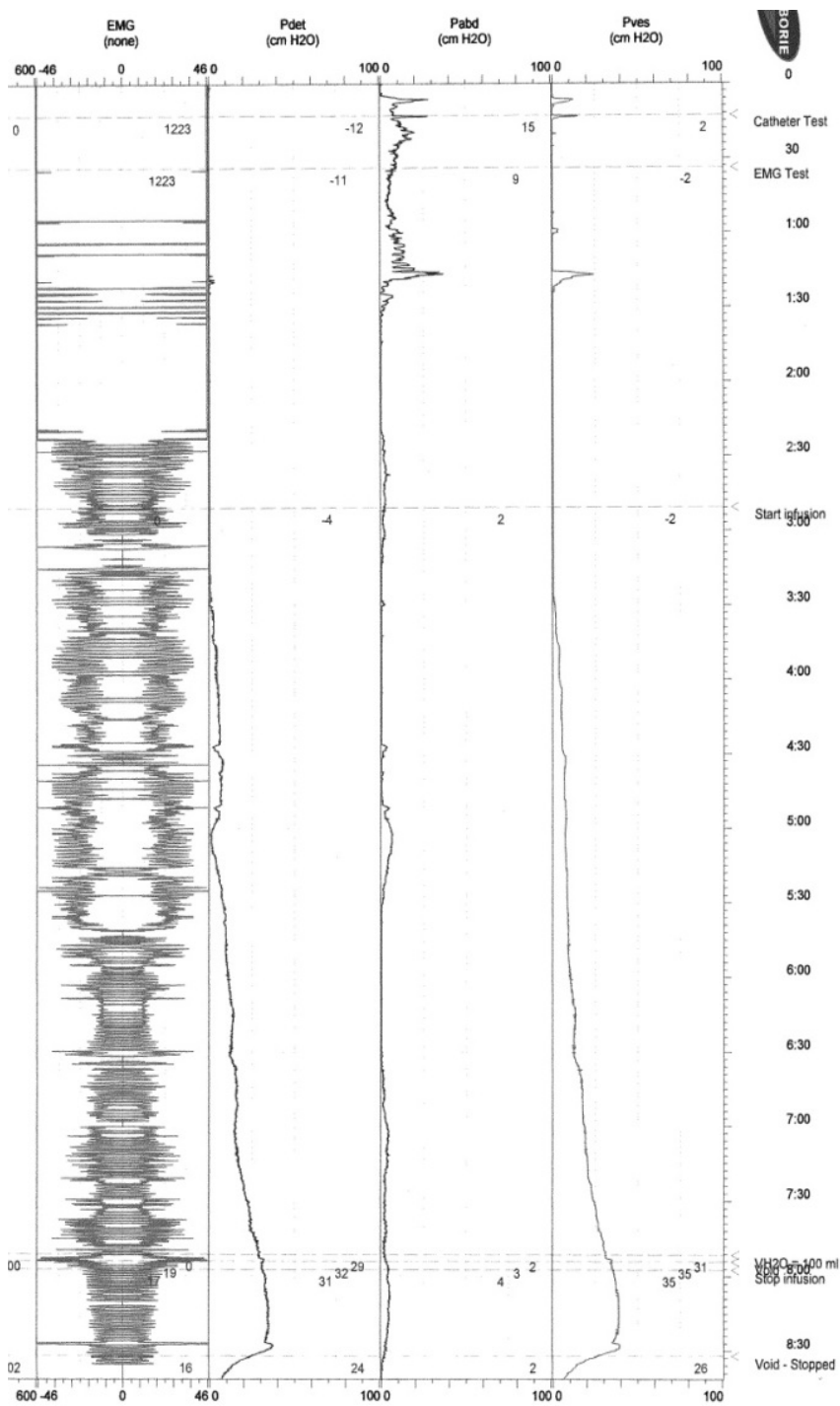


Figure 5: Compliance is significantly increased in dogs with SCI and decreases with time to be similar to controls. Boxplot of compliance values (\log_{10} -transformed) by time among 10 control dogs (00No) and 20 dogs with SCI on days 0 (00Yes), 3 (03Yes), 7 (07Yes), and 42 (42Yes). The grey boxes extend from the 25th to 75th percentiles of the data; the horizontal line with a triangle represents the median; thin vertical lines extending to horizontal lines represent a multiple of 1.75 times the 2nd (lower) or 3rd (upper) quartiles; circles with horizontal lines are outliers.

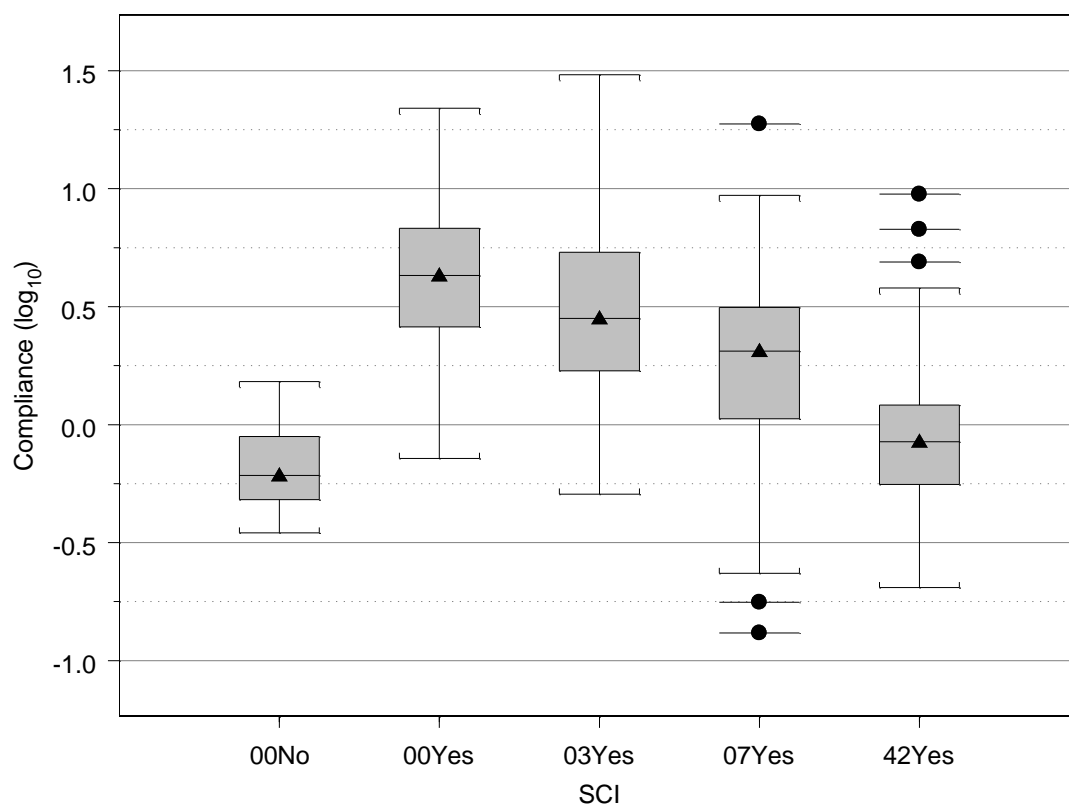


Table 1. Baseline (admission) characteristics of 88 male dogs enrolled in a clinical trial receiving either treatment 0 (n = 46) or treatment 1 (n = 42). There were no significant differences between groups for any factor. P values were determined using the Wilcoxon rank-sum test for continuous variables (1.a.) and the chi-squared test for categorical variables (1.b.), or Fisher's exact test (indicated by an *).

a. Continuous variables: median (range)

| <i>Variable</i> | <i>Group 0</i> | <i>Group 1</i> | <i>P value</i> |
|----------------------------|-----------------------|-----------------------|-----------------------|
| Age (years) | 5 (3 to 11) | 5 (2 to 12) | 0.3527 |
| Body weight (kg) | 7.25 (4.18 to 14.50) | 6.62 (3.84 to 31.0) | 0.3602 |
| Duration of injury (hours) | 18.5 (3.0 to 73.0) | 25.75 (3.5 to 58.0) | 0.2289 |
| TSCIS | 4 (0 to 10) | 4 (0 to 10) | 0.8786 |
| Urinary capacity (mL) | 83.3 (11.6 to 188.7) | 76.0 (23.3 to 244.4) | 0.9634 |
| Urinary compliance | 2.7 (0.7 to 12.4) | 2.9 (0.8 to 16.3) | 0.5330 |

Table 1. (continued)

b. Categorical variables: n (%)

| <i>Variable</i> | <i>Group 0</i> | <i>Group 1</i> | <i>P value</i> |
|------------------------------|-----------------------|-----------------------|-----------------------|
| Sex | | | 0.3489 |
| Male | 15 (33%) | 9 (21%) | |
| Male castrated | 31 (67%) | 33 (79%) | |
| Chondrodysplastic breed | | | 1.0000* |
| No | 3 (7%) | 3 (7%) | |
| Yes | 43 (93%) | 39 (93%) | |
| Dachshund | | | |
| No | 17 (37%) | 9 (21%) | 0.1736 |
| Yes | 29 (63%) | 33 (79%) | |
| MFS admission | | | 0.9994 |
| 0 | 9 (20%) | 9 (21%) | |
| 1 | 8 (17%) | 6 (14%) | |
| 2 | 14 (30%) | 14 (33%) | |
| 3 | 15 (33%) | 13 (31%) | |
| MRI | | | 0.6765 |
| No | 21 (46%) | 22 (52%) | |
| Yes | 25 (54%) | 20 (48%) | |
| T2W hyperintensity | | | 0.5900* |
| No | 1 (4%) | 2 (10%) | |
| Yes | 22 (96%) | 18 (90%) | |
| Lesion location | | | 0.1689 |
| Thoracic (T9-T13) | 13 (29%) | 21 (50%) | |
| Lumbar (L1-L6) | 14 (30%) | 7 (17%) | |
| Both | 19 (41%) | 14 (33%) | |
| Single space (e.g., T11-T12) | | | 0.4085 |
| No | 27 (59%) | 20 (48%) | |
| Yes | 19 (41%) | 22 (52%) | |
| Duration of signs < 24 hours | | | 0.0609 |
| No | 14 (30%) | 22 (52%) | |
| Yes | 32 (70%) | 20 (48%) | |
| Survival to 42 days | | | 0.7049* |
| No | 3 (7%) | 4 (10%) | |
| Yes | 43 (97%) | 38 (90%) | |

Table 2.a. Results of multivariable generalized linear modeling of factors associated with TSCIS on Day 42 among 80 dogs with intervertebral disk herniation and spinal cord injury surviving until follow-up at Day 42. The same final model was selected from forward and backward analysis. **b.** Model from a. for which treatment group was forced into the model.

a.

| <i>Variable</i> | <i>Day 42 TSCIS</i> | <i>P value</i> |
|-----------------------------|---------------------------------|----------------|
| MFS = 0, Capacity \geq 75 | 0.44 (-2.4 to 3.2) ^a | 0.7579 |
| MFS = 0, Capacity < 75 | 7.2 (3.3 to 11.1) ^b | 0.0011 |
| MFS > 0, Capacity \geq 75 | 11.9 (8.9 to 14.8) ^c | < 0.0001 |
| MFS > 0, Capacity < 75 | 13.8 (9.6 to 18.0) ^c | <0.0001 |
| Dachshund | 2.5 (1.0 to 4.0) | 0.0094 |
| Age > 5 years | 2.1 (0.7 to 3.5) | 0.0261 |

b.

| <i>Variable</i> | <i>Day 42 TSCIS</i> | <i>P value</i> |
|-----------------------------|---------------------------------|----------------|
| MFS = 0, Capacity \geq 75 | 0.5 (-2.4 to 3.4) ^a | 0.7359 |
| MFS = 0, Capacity < 75 | 7.2 (3.3 to 11.2) ^b | 0.0013 |
| MFS > 0, Capacity \geq 75 | 11.9 (8.9 to 14.8) ^c | < 0.0001 |
| MFS > 0, Capacity < 75 | 13.8 (9.6 to 18.1) ^c | <0.0001 |
| Dachshund | 2.1 (0.5 to 3.7) | 0.0101 |
| Age > 5 years | 1.6 (0.2 to 3.1) | 0.0267 |
| Group 1 | -0.1 (-1.5 to 1.3) | 0.8692 |

Table 3. Results of multivariable generalized linear model regression analysis from 80 dogs with disk herniation surviving to 42 days post-operatively. Data were modeled as \log_{10} transformed to meet distributional assumptions of the model and were back-transformed to linear scale of compliance units. Please note that the intercept value must be added to each value (e.g., estimated mean compliance on Day 42 for a Group 1 dog ≤ 7 kg would be 1.4 (intercept) + 1.4 (Dachshund) = 2.8, versus 1.4 (intercept) for a Group 0 dog ≤ 7 kg. Results for the best fitting model as well as a model not including capacity at admission are shown.

| <i>Variable</i> | <i>Day 42 log Compliance (95% CI)</i> | <i>Back transformed</i> | <i>P value</i> |
|---|---|-----------------------------|----------------|
| <u><i>Best fitting model</i></u> | | | |
| Intercept | 0.14 (0.01 to 0.26) | 1.4 (1.0 to 1.8) | 0.0414 |
| Weight > 7 kg | 0.25 (0.12 to 0.38) | 1.8 (1.3 to 2.4) | 0.0002 |
| Capacity < 75 at admission | -0.24 (-0.36 to - 0.11) | 0.6 (0.4 to 0.8)* | 0.0004 |
| Group 1 | 0.14 (0.02 to 0.27) | 1.4 (1.0 to 1.8) | 0.0272 |
| <u><i>Model fit excluding capacity at admission</i></u> | | | |
| Intercept | 0.01 (-0.11 to 0.13) | 1.0 (0.8 to 1.4) | 0.8404 |
| Weight > 7 kg | 0.28 (0.15 to 0.42) | 1.9 (1.4 to 2.6) | <0.0001 |
| Group 1 | 0.15 (0.01 to 0.28) | 1.4 (1.0 to 1.9) | 0.0366 |